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INDOPHENOL REACTION AND REDUCTION IN LIVING OVARIES OF DROSOPHILA HYDEI

(Eight figures)

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The physiological patterns of ovaries, developing follicles, and eggs of *Drosophila hydei*, as indicated by differential reduction of methylene blue and Janus green, were described in an earlier paper (Child, 1942). The present paper is a further study of physiological pattern in this material by means of the indophenol reaction and the reduction of intracellular indophenol blue with decrease of oxygen content in the medium. The indophenol reaction has commonly been used with rapidly lethal concentrations of reagents as an indicator of intracellular presence and localization of "indophenol oxidase." According to recent authors, this oxidase is apparently a component of the cytochrome-cytochrome oxidase system.[1] The data presented in this paper were obtained with concentrations of the indophenol reagents so low that intracellular formation of indophenol blue occurred in material still living and in good condition. This material was also capable of reducing intracellular indophenol blue to a colorless form in presence of the indophenol reagents, when the oxygen content of the medium was slightly decreased. Results obtained on echinoderm development with these procedures have been briefly presented elsewhere (Child, 1941). Nothing need be added here to the description of morphological developmental pattern of the ovary in the earlier paper (Child, 1942), but use of the indophenol reaction on living material and reduction of indophenol blue by the living cells require consideration.

[1] Keilin and Hartree, 1938; Stotz, Sidwell, and Hogness, 1938; Gersch, 1941.

EXPERIMENTAL PROCEDURES

Formation of indophenol blue from the reagents dimethyl-paraphenylenediamine (para-aminodimethylaniline) and α-naphthol is an oxidative reaction strongly catalyzed in protoplasms by an oxidase, with appearance of intense blue color. Both reagents are toxic or immediately or very rapidly lethal in the concentrations ordinarily used. For use with living material extremely low concentrations are necessary. For work with the ovaries the reagents, obtained from the Eastman Company—the diamine in

5 per cent solution and the α-naphthol in powdery solid form—were used as follows: One small drop (40 drops equal 1 ml.) of the diamine 5 per cent solution in 10 ml. of insect Ringer solution[2] or water, a concentration of 1 part diamine in 8,000, served as stock solution and, except in some early experiments, was made fresh for each day's or few hours' use. Stock solution of α-naphthol was 1 mg. in 10 ml. of insect Ringer or water, a concentration of 1 part in 10,000 or in some cases 0.5 mg. in 10 ml. (1/20,000), also made fresh daily. The α-naphthol dissolves slowly. These concentrations are far too high for use on the living material, and further dilutions were made as found by experiment to be necessary or desirable. For example, 1 small drop of each stock solution in 1 ml. of Ringer containing the ovaries, follicle strings, or eggs, giving concentrations of 1/320,000 diamine and 1/400,000 α-naphthol, represent approximately the upper limit of concentrations found useful; and these are often sufficiently toxic to alter the reaction. With concentrations of this order at room temperature the indophenol blue color usually becomes visible in about 5 minutes in the most rapidly reacting regions of the ovaries. Much lower concentrations give the reaction even more distinctly but less rapidly. With 1 small drop each of one-fourth the stock concentrations in 2–3 ml., 1/2,560,000–1/3,840,000 diamine and 1/3,200,000–1/4,800,000 α-naphthol, the differential is distinct and becomes clearly visible after 20–40 minutes. With 1/4,800,000 diamine and 1/6,000,000 α-naphthol it is also distinct, but appears still more slowly. Undoubtedly, still lower concentrations would give the reaction with a volume of solution sufficient to provide adequate amounts of the reagents, but thus far concentrations lower than these have not been used.

Some 1,200 ovaries have been used in this study. The ovaries are removed from flies in Ringer, as described in the earlier paper (Child, 1942); and follicle strings, single follicles, and eggs are easily isolated. One ovary of a pair is often left intact, the other more or less completely separated into follicle strings in the same preparation. In another procedure the two members of a pair, or of several pairs at approximately the same stage of development, are placed in different concentrations of reagents or under conditions differing otherwise, and the reactions compared. In other cases the reaction of advanced and young ovaries is compared in the same solution of reagents, and in still others one pair or several pairs in the same stage are placed in the same solution. Ovaries from flies without food, from flies kept in old food bottles with development of ovaries retarded or completely inhibited, and from well-fed flies with rapidly developing ovaries have served as material.

At all stages before deposition of yolk begins in the oöcyte, the follicles are almost colorless and transparent. In the more advanced follicles of these stages cell nuclei and cell boundaries are clearly visible. As yolk accumulates, the oöcyte becomes almost opaque; but follicle wall and nurse cells remain translucent, and nurse cell boundaries and nuclei are still clearly visible. Full-grown eggs are almost opaque, but differentials in formation and reduction of indophenol blue are distinctly visible. As in the earlier paper, stages of follicle development are distinguished as pre-oöcyte stages, i.e., stages in which oöcyte and nurse cells are

[2] Insect Ringer solution recommended by Beadle for work with *Drosophila:* NaCl, 6.075 gm.; KCl, 0.283 gm.; $CaCl_2$, 0.170 gm., H_2O, 1 liter; I am also deeply indebted to Dr. Beadle and Dr. Tatum for providing a stock of flies and for food bottles.

not visibly different; early-oöcyte stages, with growth of oöcyte and yolk formation beginning; $\frac{1}{4}$-, $\frac{1}{3}$-, $\frac{1}{2}$-oöcyte stages, in which the oöcyte is from one-fourth to one-half the length of the follicle; and advanced-oöcyte stages, with oöcyte occupying most of the follicle and only a few nurse cells or only debris anterior to it. In the full-grown egg all remains of nurse cells have disappeared, and the long chorionic appendages have developed, though the separation into four filaments has not been observed until after isolation of eggs.

Except in cases concerned with the possible effects of longer isolation periods preceding reaction, reagents are added as desired as soon as possible after isolation, rarely more than 5–10 minutes. The volume of solution used is definite, usually 1–3 ml.; and uniform concentration is maintained during the reaction period by frequent stirring or by currents from a dropper. That the reaction patterns observed are not merely patterns of differential penetration of one or both of the reagents is shown or indicated in various ways: first, gradient patterns are in the same direction, with a wide range of concentrations of reagents, but are altered, reversed in direction, or obliterated by altered physiological and experimental conditions; second, by placing ovaries in one of the reagents for a few minutes, then adding the other in concentration high enough to give the reaction rapidly, it becomes evident that the first is present in all the colorless follicles, but the reaction gradient pattern is the same as when both reagents are added at the same time. In the large advanced follicles penetration of reagents into the interior of the large mass of nurse cells may require some time, but the follicle gradient is evident in the superficial regions. In the full-grown eggs entrance of the reagents is probably retarded by the chorion, but that the reaction pattern of the egg represents merely a chorionic pattern appears at least improbable in the light of the data on earlier stages.

The reaction patterns are not essentially altered by differences of five or six times in concentrations of the two reagents provided they are below the range of rapid toxic effects. With increasing concentrations both reagents become sufficiently toxic to retard the reaction, to reverse direction of the gradient pattern completely or in part in consequence of differential susceptibility, or even to obliterate it. Effects on the reaction pattern of various concentrations of KCN and the reaction in ovaries killed by long standing in Ringer after isolation, by boiling water, by distilled water, by KCN, and by $HgCl_2$ have also been observed.

A slight decrease in the oxygen content of the medium containing reagents and ovaries is sufficient to bring about the differential reduction of intracellular indophenol blue to a colorless form, and on readmission of oxygen color returns. Differential decoloration and recoloration can often be brought about more than once in the same ovaries before their ability to reduce is lost with approach of death. By sealing the ovaries in the reagent–Ringer solution in small cells gradual decrease in oxygen content of solution and differential reduction are brought about by oxygen uptake of the ovaries, as in reduction of methylene blue and Janus green (Child, 1942). For reduction a considerable number of ovaries or of isolated eggs has been used, and testes or other tissues have often been added in order to bring about reduction rapidly and avoid, as far as possible, toxic effects of reagents or indophenol blue or

long isolation. The tendency of eggs to stick to glass when first removed decreases, and in sealed preparations most of them or the added tissues can be moved about by change in position of the slide with respect to gravity in order to avoid local differences in oxygen content. With long-continued reaction and intracellular accumulation of indophenol blue the ability to reduce decreases and reduction is retarded, the reduction gradient is reversed in direction, or reduction does not take place in certain or all parts. With deterioration in condition, regions which have reduced may show more or less reoxidation in the sealed preparation without any increase of oxygen content.

Figures of developmental stages appear in the earlier paper (Child, 1942). Figures of the present paper are diagrammatic outlines with general directions of progress of reaction, reduction, and reoxidation indicated by means of arrows. They are drawn from ocular-micrometer measurements of ovaries, follicles, and eggs. Figures 1–5 and 7–8 are drawn to the same scale, approximately ×150, as figures of earlier ovarian stages and single follicles in the earlier paper; Figure 6, like the earlier figures of more advanced stages, is drawn to half that scale (×75).

THE "NORMAL" REACTION PATTERN

Ovaries of flies provided with fresh food on emergence grow and develop rapidly. Such ovaries are believed to be in good physiological condition and to constitute the most favorable material for determination of what may be regarded as the normal reaction pattern. However, since the reaction pattern remains the same with a wide range of low concentrations of reagents, but is altered, even in these ovaries, in concentrations above this range, with indications of relatively early toxic effect, the reaction pattern observed with the low concentrations in ovaries of well-fed flies is regarded as the normal pattern. In general, concentrations of 1/640,000 diamine and 1/800,000 α-naphthol and all lower concentrations show this pattern until secondary effects become evident. With concentrations twice these, the same pattern may appear in earlier stages of reaction; but with further reaction, evidence of differential toxic effect usually appears in retardation of further reaction in the earliest follicles, while the less susceptible later developmental stages and full-grown eggs may still show normal pattern. For alteration of pattern in these, still higher concentrations or further progress of reaction is necessary.

In the normal reaction pattern the elongated terminal apical regions of the follicle strings and the most apical follicles show the reaction first, and it progresses basipetally from follicle to follicle, both in intact ovaries and in isolated follicle strings. In the youngest ovaries with only four or five follicles and in corresponding regions of more advanced ovaries the basipetal gradient in appearance of indophenol blue is clearly distinguishable, but the differential is not great because all follicles in these ovaries or regions are in very early developmental stages. In the older ovaries the basipetal differential is much greater; in those with full grown eggs in the basal region the reaction is far advanced in colorless follicles before it is visible in late oöcytes or full-grown eggs.

The time of first appearance of reaction differs with concentration of reagents. For example, with diamine 1/640,000 and α-naphthol 1/800,000, reaction is usually visible in the most apical follicles of isolated strings in good condition in about 5 minutes and in apical regions

of young intact ovaries 2–3 minutes later. It becomes visible over the whole length of early short follicle strings and young ovaries (Fig. 1) and in corresponding regions of more advanced ovaries (Figs. 2 and 3) in 10–20 minutes. In advanced ovaries with late oöcytes and full-grown eggs these usually show reaction only after 35–45 minutes. With concentrations half of the above, appearance of reaction apically requires about twice the time, but later basipetal progress of reaction does not differ greatly from that with the higher concentration. With increase in concentration of agents reaction becomes more rapid until early toxic effects begin to appear.

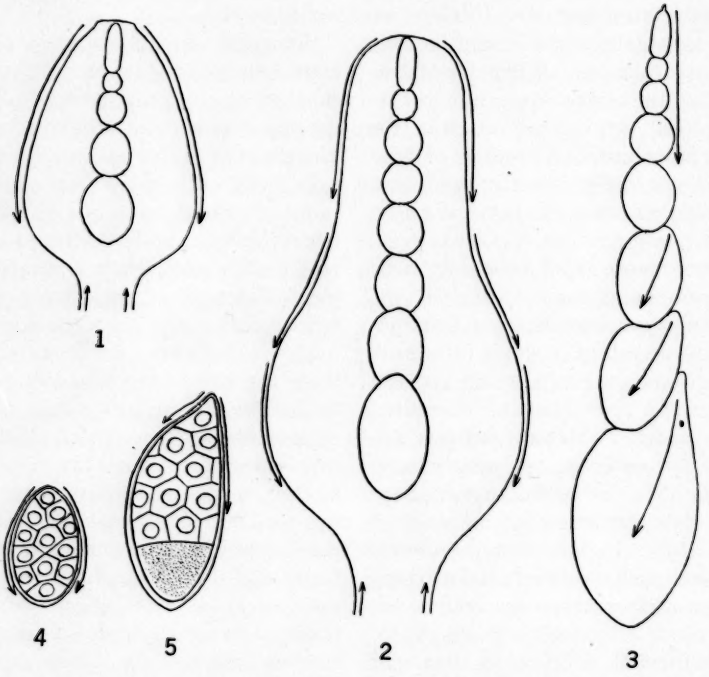
A reaction gradient has not been observed with certainty in single follicles of the earliest stages, probably on account of their small size; but the elongated apical region from which the follicles constrict off successively apparently does show a slight basipetal reaction gradient with very low concentrations of reagents in some cases. In the later pre-oöcyte follicles and early-oöcyte follicles basipetal progress of reaction is usually visible with the lower concentrations, as indicated in the fifth follicle from the upper end of Figure 3 and in Figure 4. In early-oöcyte and later follicles, sometimes in later pre-oöcyte follicles, a ventrodorsal reaction component is often visible, as indicated in the two follicles at the lower end of Figure 3 and in Figure 5. The prospective ventral side of the egg is toward the outer surface of the ovary, and in these follicles this side apparently reacts a little more rapidly than the other. This ventrodorsal differential has not been observed in all possible cases in isolated follicles and strings but does appear to be characteristic of reaction in the lower concentrations. In intact ovaries it is usually distinct, but its significance there is doubtful because the prospective ventral side is more directly exposed than others. However, more rapid reaction on the dorsal side has not been seen in follicles of these stages, and the ventrodorsal differential is at best slight and readily obliterated.

Reaction is more rapid in single isolated follicle strings than in follicles of the same stages in intact ovaries. The elongated apical region of the ovarian tubule and the four or five earliest follicles separate from each other less readily than more advanced follicles. The later pre-oöcyte follicles and all later stages separate readily and, when separated as single follicles, later pre-oöcyte, early-oöcyte, follicles, and often the nurse cells of $\frac{1}{2}$-oöcyte follicles, react more rapidly than the same stages in follicle strings and often more rapidly than the apical regions of isolated strings. Isolated follicle strings are somewhat more exposed to the reagents than when in intact ovaries, but single isolated follicles are not appreciably more exposed than when connected in strings. In short, the reactions of isolated strings and even more those of isolated single follicles suggest that oxidase activity is increased by isolation, as if a stimulation or irritation resulted from it. It is, of course, a familiar fact that increase of oxygen uptake and CO_2 production usually follow experimental separation of parts by section in many invertebrates; and that isolation of follicles and perhaps of follicle strings, has a similar effect, involving indophenol oxidase, seems not improbable.

The torn end of the oviduct reacts almost, or quite, as early as the apical region of the ovary, and the reaction progresses from the torn end in a short gradient (Figs. 1 and 2). This gradient does not extend to the basal follicles, and the region between it and these follicles re-

acts as late as, or later than, the basal follicles. Like the more rapid reaction of isolated follicle strings and follicles, this reaction gradient from the torn end of the oviduct also suggests an increase in oxidase activity, resulting from the injury. Reduction of methylene blue and Janus green also occurs early at the torn end of the oviduct and shows a gradient like that of the indophenol reaction.

Thus far, only the time factor in appearance of the reaction has been considered. There is, however, another factor, the differential in intensity or depth of color, apparently dependent on intracellular concentration of indophenol blue. In the early stages of reaction, depth of color decreases basipetally in the normal pattern; but with further progress of reaction it may appear equal at all levels down to the most advanced colorless follicles or parts, which may range from early-oöcyte to nurse cells of ½-oöcyte follicles, according to stage of ovary and nutritive conditions (Child, 1942); and sooner or later the older color-



FIGS. 1–5.—Outlines (approximately ×150) drawn from ocular micrometer measurements of ovaries, isolated folicle strings, and single follicles, with directions of progress of "normal" differential indophenol reaction pattern indicated by arrows; the longer arrows in Figs. 1 and 2 indicate regions of more rapid progress of reaction. Fig. 1, ovary from recently emerged fly, one follicle string shown, reaction gradient basipetal. Fig. 2, a later developmental stage with basipetal reaction gradient indicated. Fig. 3, isolated follicle string from a rapidly developing ovary with general basipetal gradient and in the more advanced follicles a basipetal follicle gradient with a slight ventrodorsal component. Fig. 4, an isolated pre-oöcyte follicle with basipetal reaction gradient. Fig. 5, a more advanced follicle with growing oöcyte; reaction basipetal and slightly ventrodorsal. These figures serve equally well to indicate the normal reduction gradient.

less follicles may appear more deeply colored than the youngest. This secondary differential is real, not merely the result of basipetal increase in size of follicles and light transmission through a greater thickness of tissue. It is evident in tissue layers of equal thickness. However, with sufficient quantity of reagents the small youngest follicles finally become as deeply colored as the older.

With extremely low concentrations of indophenol reagents the earlier reaction is usually followed by a gradient of decrease of color in the younger regions of the ovary or isolated follicle string. The most apical regions often become almost colorless, although showing considerable color at an earlier stage of reaction. This loss of color has been seen only in the youngest five or six follicles, and it decreases basipetally in this region. It occurs while the reagents are present and reaction is progressing in regions farther basal and is not a result of low oxygen content of the solution. Loss of color does not involve loss of ability to react, for with increase in concentration of agents these follicles show renewed progress of reaction. Moreover, even without addition of reagents the partial decoloration may be followed later by gradual recoloration in the original solution, apparently as physiological condition changes in the course of time following isolation. The observations suggest that the youngest follicles are able, or become able, in consequence of excitation or irritation by the reagents or the indophenol, to reduce irreversibly or otherwise dispose of minute amounts of indophenol blue. If this is the case, this ability decreases basipetally in the ovaries with progress of development of follicles and gradually decreases and disappears after isolation, presumably with change in condition as death approaches. Apparently this decoloration is not the reversible reduction which occurs in low oxygen, for color does not return with increase of oxygen content of the solution. Moreover, as will appear later, reoxidation after reduction in low oxygen often gives less color in apical regions than before reduction in low concentrations of reagents. Similar gradual decoloration of intracellular methylene blue and Janus green by the younger follicles of living ovaries in low dye concentrations, frequently renewed and without decrease of oxygen content, has been noted (Child, 1942).

In the light of the alterations and reversals in direction of the reaction gradient under experimental conditions, described in the following section, it appears possible that the apparent retardation of reaction with a gradient decreasing basipetally in the apical regions may result from a basipetally decreasing differential susceptibility in the younger ovaries and apical regions of older ovaries to toxic action of one or both reagents or to indophenol blue. However, the decoloration in the younger follicles with very low concentrations of reagents seems to be an expression of activity of the living cells. If this is true, the apparent retardation of reaction, as indicated by depth of color, may also be an expression of this activity in concentrations of reagents high enough to prevent actual loss of color. The cells may dispose of some of the indophenol blue, but the reaction progresses more rapidly than the decomposition, so that the result is merely an apparent retardation rather than an actual decoloration. That the reaction may be differentially retarded experimentally will appear in the following section, but at present it seems impossible to determine whether the apparent differential retardations of primarily normal reactions are actual retardations re-

sulting from differential toxic action of reagents or indophenol or, like the differential decoloration, perhaps expressions of the differential ability to decompose the indophenol blue in some way, but not rapidly enough to keep pace with its formation. Both these factors may perhaps be concerned in some cases.

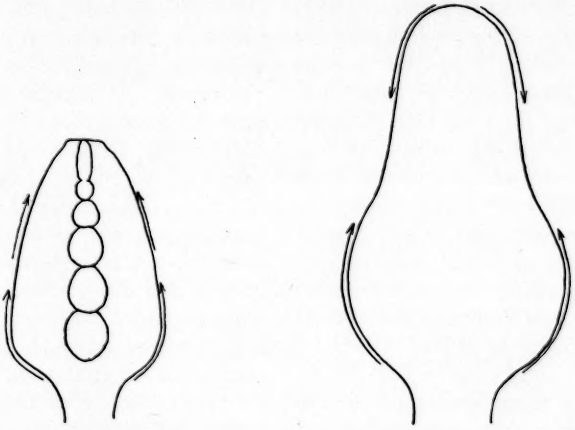
FIG. 6.—Outline of full-grown egg seen from side (approximately ×75), with progress of reaction posteriorly and dorsally indicated. The second reaction gradient, observed only in small percentage of eggs, is also indicated. The normal reduction pattern is similar to the reaction pattern.

In late stages of oöcyte development with some remains of nurse cells still present anteriorly within the chorion these show the reaction before the oöcyte. In the isolated oöcyte of these stages and in the full-grown isolated egg the characteristic reaction gradient is from the anterior end posteriorly. In many eggs viewed from the side the ventral side appears to be slightly in advance of the dorsal, the reaction progressing somewhat ventrodorsally as well as longitudinally (Fig. 6). However, these reaction gradients in the egg are slight; even the longitudinal gradient is not always distinguishable with certainty. Variable percentages of full-grown eggs from different ovaries, usually not more than 25–30 per cent in any lot, often only a few, show a second reaction gradient from the posterior end, extending over about the posterior fourth. In advanced intact ovaries the basal series of eggs with posterior ends at the base of the ovary close to the ovarian end of the oviduct usually show this secondary reaction gradient from the posterior end, but it has not thus far been observed in eggs of a second zone apical to the basal zone in these ovaries. Whether it is associated with exposure of the posterior ends of the basal eggs to conditions different from those farther apically in the ovary, or with some other factor, it is evidently not a constant or characteristic feature of egg pattern. The data on full-grown eggs indicate the presence of a longitudinal reaction gradient with the high end anteriorly as the most conspicuous feature of the reaction pattern, though not always clearly distinguishable, with a slight ventrodorsal differential visible in many eggs; this pattern is the same as that of the ovary and follicles.

ALTERATIONS OF REACTION PATTERNS

Nutritive conditions.—When flies are kept after emergence in the same food bottles as the eggs, larvae, and pupae from which they devoloped they may live for several weeks, but their ovaries are more or less completely inhibited in development. In some stocks on old food, ovaries show almost no advance in development from the stage at emergence; in others they may develop very slowly; but in no case seen have they attained maturity or even the stage of yolk deposition in the oöcytes.

Ovaries or isolated follicle strings from flies kept on old food for more than 2 or 3 days after emergence almost always show more or less complete reversal of longitudinal reaction gradient pattern. In ovaries with little or no growth and development after emergence the reversal is usually complete; i.e., the reaction becomes visible first in the most basal follicles and progresses acropetally (Fig. 7). In these cases the basipetal reaction gradient may appear earlier or later than, or at the same time as, the acropetal and either may be the longer or both may be equal in length. In these cases the normal basipetal reaction gradient either has not been completely obliterated or has reappeared apically. On the other hand, a normal reaction pattern has been observed with low concentrations of reagents in some ovaries from stocks kept 2–3 weeks on old food, but with some growth and development. As might be expected, "old food," i.e., food in which the preceding generation laid eggs and the resulting larvae developed, evidently provides more adequate nutrition in some cases than in others.



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FIGS. 7 AND 8.—Outlines of ovaries (approximately ×150) indicating altered reaction patterns. Fig. 7, early developmental stage with reversed longitudinal gradient. Fig. 8, later stage with two opposed reaction gradients.

One stock of flies showed complete reversal of the longitudal reaction gradient in various low concentrations after 24 hours on old food following emergence. In some ovaries from old food stocks two opposed reaction gradients appear, one basipetal from the apical region, the other acropetal from the basal follicles (Fig. 8). These partial reversals have been observed in some ovaries from flies kept on old food only a few days but are apparently most frequent in ovaries which have undergone some growth and development on old food, as in Figure 8.

Flies kept without food in bottles with a ball of wet absorbent cotton to keep the air moist have lived at most only a few days. In a stock under these conditions more than half were dead after 4 days. In four lots of ovaries (thirty-two

in all) from flies of this stock, after 4 days' starvation there was complete reversal in thirty and almost complete reversal with very slight basipetal gradient in the extreme apical region in two. Different concentrations of reagents were used in each of these lots, ranging from 1/2,880,000 to 1/160,000 diamine and from 1/2,400,000 to 1/200,000 α-naphthol, with complete reversals in both lowest and highest concentrations. Ovaries from two females still alive after 5 days without food showed complete reversal in concentrations of 1/4,800,000 diamine and 1/6,000,000 α-naphthol with one pair, 1/3,840,000 diamine and 1/4,800,000 α-naphthol with the other, the first traces of reaction becoming visible only after 40–45 minutes. These concentrations are the lowest used in any experiments and are mentioned here as evidence that reversal of the reaction gradient is not due to differential toxic effect of high concentrations of reagents.

These reversals in direction of the reaction gradient associated with inadequate nutrition or complete starvation suggest a differential susceptibility decreasing basipetally and involving activity of the oxidase. This may conceivably be differential susceptibility either to the nutritive conditions or to one or both reagents in consequence of the nutritive conditions. According to the first of these possibilities, the nutritive conditions are the differentially inhibiting factor, their effect decreasing basipetally from the apical regions where the oxidase activity is normally greatest. The alternative possibility is that the ovaries of inadequately nourished or starved flies become so highly susceptible to toxic action of external agents that even the extremely low concentrations of one or both of the indophenol reagents are toxic to the reaction with a gradient decreasing basipetally. The data do not permit decision between these alternatives. Contractions of the ovaries continue even after the reaction is clearly visible. The indophenol reagents do become toxic in sufficiently high concentrations, as the following section will show, but that the extremely low concentrations with which reversals appear in starved animals are appreciably toxic during the period necessary for reaction seems improbable. On the other hand, it seems much more probable that under nutritive conditions which permit little or no growth and development of ovaries oxidase activity may be considerably decreased, with a differential decrease from the regions where activity is normally greatest.

Change to fresh food of stocks which have shown reversal of the reaction gradient on old food is followed by rapid growth and development of ovaries and reappearance of normal basipetal reaction gradient with the same ranges of concentration that gave reversal on old food. The length of time necessary for return of normal reaction apparently differs somewhat in different stocks, and exact determination has not been attempted. After 2 days on fresh food the reaction gradient is usually normal. After 4–5 days on fresh food stocks in which the ovaries remained small and in early stage of development, and showed reversed gradient on old food, have large ovaries in advanced stages of development and show normal reaction gradients.

Effects of high concentrations and of reagents used separately.—Different concentrations of mixtures of indophenol reagents can be so chosen that of two lots of ovaries from the same flies, one member of each pair in each lot, the one in low concentrations will show normal reaction gradient, the other in high con-

centrations, complete reversal. The critical range of concentration varies with physiological condition of ovaries, and within that range there is often variation in ovaries of a single lot in approximately the same developmental stage. Some may show normal gradient; others a reversed gradient; still others two opposed gradients. Further experiments have shown that α-naphthol is chiefly responsible for the reversals with increase of concentrations. The following series will serve as examples of a result obtained quite consistently with ovaries from flies in good nutritive condition.

1. Young ovaries, slightly more advanced than Figure 1, from ten flies were separated into two lots, one ovary from each fly in each lot. In a mixture of diamine 1/2,560,000 and α-naphthol 1/3,200,000 all showed an early stage of normal reaction gradient in from 45 minutes to 1 hour. Their mates in a mixture of diamine 1/640,000 and α-naphthol 3/800,000 all showed complete reversal in 25 minutes.

2. The follicle strings of one large advanced ovary of a pair, with two zones of full-grown eggs basally, were separated and subjected to a mixture of diamine 1/2,560,000 and α-naphthol 1/3,200,000. Normal reaction gradient down to the full-grown eggs was evident in from 45 minutes to 1 hour. The eggs showed no reaction up to that time. Later an apparent retardation of reaction, decreasing basipetally, appeared in the apical three to five follicles (see p. 147), while in regions farther basal the reaction progressed.

The separated follicle strings of the other ovary of the pair in a mixture of diamine 1/640,000 and α-naphthol 1/400,000 all showed reversed reaction gradient above the full-grown eggs. Reaction in the eggs was not observed; but, in general, reaction in eggs is not reversed in concentrations of this order.

These reversals of the reaction gradient in relatively high concentrations indicate a differential susceptibility to toxic action with inhibition or retardation of reaction greatest in the youngest follicles and decreasing basipetally. The reversals may become very distinct, with gradation from intense reaction in the basal follicles to a mere trace of reaction apically.

Attempts to determine whether reversals in the higher concentrations are due chiefly or wholly to one of the reagents have been made by subjecting ovaries to various concentrations of one reagent for different periods before addition of the other. These experiments indicate that both reagents are toxic to the indophenol reaction in sufficient concentration but with different effects. The reagent chiefly responsible for reversals of the general ovarian reaction gradient is α-naphthol.

3. Medium young ovaries, one-third to one-half the length of the most advanced, in α-naphthol 1/400,000 for 2 hours before addition of diamine 1/320,000 all showed normal reaction gradient. Another lot from the same stock in α-naphthol 2/400,000 for 2 hours before addition of diamine 1/300,000 were normal, except one with slight reversal apically. A third lot from the same stock in α-naphthol 3/200,000 for 2 hours before addition of diamine 1/320,000 were all reversed in earlier stages of reaction, but the reversed gradients gradually became less distinct until the color appeared uniform at all levels, and after 45 minutes there seemed to be a slight decrease basipetally in depth of color. This progressive obliteration of the reversed reaction gradient, perhaps with a slight basipetal gradient, probably results from progressive retardation of further reaction in the more basal regions by the accumulated indophenol blue. With still higher concentrations of α-naphthol, secondary reversal of the reversed gradient has not been observed.

4. In other lots in various developmental stages with α-naphthol 1/100,000 and 1/80,000 and with addition of diamine 1/1,280,000 or 1/320,000 after 15–30 minutes, reaction was almost completely inhibited in the younger follicles. The youngest ovaries and corresponding apical regions of more advanced ovaries reacted only very slowly and slightly, and in the latter the reaction increased basipetally. Controls in diamine 1/1,280,000 and α-naphthol 1/1,600,000 all showed normal reaction gradients.

The reversals of the reaction gradient by α-naphthol concern the follicle strings as wholes, but the less susceptible older follicles and full-grown eggs may still show normal longitudinal gradients.

In diamine alone the colorless follicles and the nurse cells of older follicles become yellow or, in high concentrations, brown. This color change is most rapid and greatest in the most advanced follicles with largely intact nurse cells (e.g., $\frac{1}{3}$- and $\frac{1}{2}$-oöcyte follicles), decreases in rate and intensity acropetally in the follicle strings, and is very slow and slight or not evident at all in the youngest follicles. It occurs within an hour in diamine 1/640,000 and within 2 minutes in the older follicles with diamine 3/320,000. Even when naphthol, as well as diamine, is present, the more advanced follicles often become more or less yellow before the indophenol reaction appears. Although the color change is most rapid and greatest in the most advanced colorless follicles and the nurse cells of oöcyte follicles, it does not reverse the indophenol reaction gradient in the whole ovary or follicle string, though it may retard reaction. After addition of α-naphthol the reaction gradient is normal unless reversed by other conditions, but the color is greenish rather than pure blue. However, in early-oöcyte to $\frac{1}{2}$-oöcyte follicles in which the nurse cells are most deeply colored the oöcyte may react before the nurse cells after they have become deep yellow.

The significance of this change in color is not clear. It seems to represent a reaction of some sort with cell constituents that increase in amount with progress of development, particularly in the nurse cells. Whether the change in color indicates death is not known, but it is not necessarily associated with evidence of cytolysis or other change in appearance of the cells.

The effect of KCN.—Physiological gradients are, in general, differentially susceptible to cyanide, the susceptibility to strongly inhibitory or gradually lethal concentrations decreasing from higher to lower levels of these gradients. Also, indophenol oxidase has been shown to be cyanide-sensitive. In view of these facts, it seemed of interest to examine effects of cyanide on the indophenol reaction in these ovaries. For this purpose KCN was made up in insect Ringer, and ovaries were subjected to various concentrations for various periods from a few minutes to several hours. Some lots were washed and brought into fresh Ringer before the indophenol reagents were added; in others the indophenol reagents were added to the KCN solution, either immediately or after some time in KCN alone. Preliminary experiment showed that KCN concentrations from M/1,000 to M/2,000 were most useful; and in all except two of thirty-one lots, including more than a hundred ovaries of all stages, diamine 1/1,280,000 and α-naphthol 1/1,600,000 were used because with these low concentrations there is no evidence of toxic effect within the period necessary for reaction, and in control ovaries in good condition the reaction gives a distinct color gradient in sufficiently short time, about 20 minutes, for purposes of comparison. The following summary of experiments gives the chief results.

In KCN M/500 the cells of the youngest follicles are cytolyzed, and even the much less susceptible eggs usually disintegrate within the chorion or burst in an hour. Basipetal gradients of cytolysis or disintegration have been seen with this concentration. With addition of indophenol reagents to the KCN solution after 10 minutes there is scarcely a trace

of reaction at any time. On the other hand, in KCN M/5,000 with indophenol added to the KCN solution after 5 minutes the reaction is retarded; color becomes visible after 40–45 minutes instead of 20 minutes, as in controls, but the gradient is normal in direction. The reaction is progressively inhibited, only earlier stages of coloration appear, never the deep blue of controls; and the gradient is always slight. Ovaries washed and reagents added in Ringer after 25 minutes in KCN M/5,000 show similar retardation of reaction with slight normal gradient, but the reaction is not completely inhibited in early stages, as it is with continuous exposure to this concentration of KCN.

Different lots of ovaries in various developmental stages in KCN for 30 minutes, then washed and reagents added, show retardation of reaction, very slight normal longitudinal gradient, no gradient, or slight reversed gradient in the colorless follicles, and usually no definite gradients in single follicles; also, color never attains the depth of controls. Full-grown eggs are little or not at all affected.

Young ovaries in KCN M/2,000 continuously with reagents added after 1 hour show retardation of reaction, no gradient, or distinctly reversed gradient, and inhibition of reaction, with color less deep than in controls at all levels.

Ovaries of middle developmental stages with most advanced follicles in $\frac{1}{3}$- to $\frac{1}{2}$-oöcyte stages in KCN M/2,000 continuously with reagents added after $2\frac{1}{4}$ hours all show reversed longitudinal gradient, with reaction almost completely inhibited apically but moderately deep basally, though never as deep as controls. In most of the advanced follicles the oöcyte reacts before the nurse cells; in others there is no distinguishable difference.

With similar exposure periods to KCN M/1,000 and the same concentrations of indophenol reagents, results are in general similar to those with M/2,000 but more extreme. The first appearance of color is more retarded; the longitudinal gradient is reversed, with little or no color apically, with oöcytes reacting before nurse cells in the more advanced follicles, and with complete inhibition of reaction at an earlier stage than with lower concentrations.

Results with KCN are definite and consistent and indicate a differential susceptibility to KCN, decreasing basipetally, as might be expected. The indophenol reaction is inhibited to such a degree that the normal longitudinal gradient is obliterated or reversed with the higher concentrations, and oöcytes of the more advanced follicles react before nurse cells. Ventrodorsal follicle gradients are, at best, slight and are apparently obliterated by KCN.

Less attention has been given to the effect of KCN on full-grown eggs, but it is evident that the reaction in these is much less susceptible than in earlier developmental stages. With KCN M/2,000 and M/1,000, eggs show normal gradients less frequently; and in some lots progress of reaction from the posterior end seems to be much more frequent than in controls. These data are perhaps indicative rather than conclusive; but, as regards earlier stages, there can be no doubt that KCN inhibits the indophenol reaction differentially in the longitudinal ovarian axis and that, in consequence of the differential inhibition, reaction gradients may be obliterated or reversed in direction.

THE REACTION IN DEAD OVARIES

It has not been determined with certainty how long isolated ovaries live in

Ringer. Contractions have been seen up to 2 hours after isolation, but visible changes in cells of the younger colorless follicles have been observed only after longer isolation. Apparently ovaries isolated in insect Ringer remain alive and in good condition at least several hours. Ovaries that have apparently died in Ringer are yellowish in color, and the cytoplasm in colorless follicles is usually more granular than in fresh ovaries or appears to be aggregated in irregular masses. With the addition of indophenol reagents such ovaries gradually become uniformly grayish blue, rather than the brilliant blue of fresh material, and finally appear almost or quite opaque. As already noted, it is uncertain whether the yellow or brown color produced by diamine indicates death; when, and where it becomes deep, indophenol reaction is retarded, and the color is more like that in dead than in living ovaries.

With intense indophenol reaction and deep blue, change in color from pure blue to purple occurs frequently in $\frac{1}{3}$- to $\frac{1}{2}$-oöcyte follicles, usually first in the nurse cells and in full-grown eggs; but the younger follicles remain blue, at least for much longer time. This color change is apparently an effect of intracellular indophenol blue and may be associated with death or approach of death in the older follicles and eggs.

In KCN M/500 ovaries are apparently killed in $\frac{1}{2}$ hour or less. If indophenol reagents are added only 5 minutes after exposure to KCN, there is little reaction. With addition of indophenol reagents after 25 minutes in KCN M/2,000 only a faint grayish blue appears gradually and uniformly in young and old follicles and full-grown eggs. With higher concentrations of reagents the gray-blue becomes somewhat deeper. Apparently in these cases there is little or no catalytic effect of oxidase.

Ovaries killed in boiling water become yellow, and after the addition of indophenol reagents a uniform grayish blue color appears in 2–3 minutes. In diamine 1/960,000 and α-naphthol 1/1,200,000 color remains slight, but with further addition of reagents it becomes uniformly deeper; i.e., the depth of color in these killed ovaries depends on the concentration of reagents. In living ovaries it is independent of concentration over a very wide range.

Ovaries placed in distilled water shorten, and the younger become almost spherical and more transparent than in Ringer. After 15–30 minutes follicle contents appear granular and somewhat yellow. Addition of indophenol reagents at this time gives only a slight, uniform grayish blue in a few minutes with no progressive increase in depth of color unless concentrations of reagents are increased. After 24 hours in distilled water the reaction is similar.

In $HgCl_2$ ovaries become almost opaque. With addition of reagents after 10 minutes in $HgCl_2$ and three washings in Ringer they become uniformly purple; but, if the $HgCl_2$ is more completely removed, the usual grayish blue of dead ovaries appears.

In these killed ovaries the reaction gradients characteristic of living ovaries are entirely absent, and the slight reaction is perhaps due to adsorption on surfaces in the protoplasm rather than to the oxidase.

DIFFERENTIAL REDUCTION AND REOXIDATION OF INDOPHENOL BLUE

Experiments on differential reduction by living ovaries, twenty-five series, including one hundred and fifteen ovaries, have been chiefly concerned with ques-

tions of the patterns of reduction and reoxidation in relation to the reaction pattern and the physiological condition of ovaries. In ovaries in good condition, with low concentrations of indophenol reagents and before the reaction becomes advanced with deep color, reduction, like reaction, progresses from the apical region basipetally in young ovaries and in the colorless follicles of older ovaries. In early-oöcyte and later stages of follicle development basipetal progress of reduction is often visible in the single follicle, and in some follicles the ventral side apparently reduces a little more rapidly than the dorsal. In short, as might be expected, the normal reduction pattern is like that of methylene blue and Janus green (Child, 1942). However, if the reaction is permitted to continue to an advanced stage with deep color in ovaries with normal reaction gradient, the reduction gradient may be more or less completely reversed in direction, with apical regions reducing last or not at all; or in extreme cases there is no reduction at any level. These reversals or obliterations of the reduction gradient apparently result from toxic action of intracellular indophenol blue. Figures 1–5, indicating the normal course of reaction, serve equally well to indicate the normal course of reduction; but gradient patterns are not always visible in single follicles in either case.

Intact regions of oviducts reduce very slowly; but adjoining the torn ends reduction is very rapid, occurring about the same time as in the apical region of the ovary, with progress toward the ovary in a short gradient like the indophenol reaction gradient and the reduction gradient with methylene blue and Janus green from the torn ends (p. 145) and, like those, suggesting stimulation or activation by the injury. Other regions accidentally injured also show rapid reduction.

A few examples concerned with the general longitudinal gradient are given:

1. Eight young ovaries from recently emerged flies in diamine 1/1,280,000, α-naphthol 1/1,600,000. In these low concentrations reaction is slow.

40 minutes: color visible apically.
70 minutes: distinct normal reaction gradient.
1½ hours: sealed in vaseline ring with small amount of solution without air bubbles.
2 hours: apical regions almost colorless, color increasing basipetally.
4 hours: apical region showing slight recoloration in sealed preparation; basal regions completely or almost completely reduced.
24 hours: all uniform gray-blue in sealed preparation.

Here reduction, like reaction, progresses basipetally; but later recoloration begins apically without readmission of oxygen and progresses basipetally until all parts show the uniform gray-blue characteristic of dead ovaries.

2. One pair of young ovaries in diamine 1/960,000, α-naphthol 1/1,200,000.

10 minutes: sealed without air in small amount of solution.
20–45 minutes: reaction basipetal.
1–1¼ hours: reduction becomes complete apically while slight color still present basally.

3. Two advanced ovaries in diamine, 1/480,000, α-naphthol 1/600,000; sealed at once in small amount of solution.

¼–½ hour: reaction only in colorless follicles with normal basipetal gradient, reduction now becoming evident in apical region. Now opened; basipetal recoloration of reduced region and further coloration of more basal developing follicles in 2–3 minutes.

4. Five ovaries, three advanced, two in middle stages; two of the larger separated into strings; diamine 1/480,000, α-naphthol 1/1,200,000.

¼ hour: color appearing apically with basipetal reaction gradient.
½ hour: sealed in small amount of solution.
1 hour: reduction basipetal, complete in apical regions.

5. One advanced ovary in diamine 1/960,000, α-naphthol 1/1,200,000.
½ hour: basipetal reaction gradient in colorless follicles; sealed in small amount of solution.
1 hour: basipetal reduction.
1½ hours: reduction complete in colorless follicles; now opened and slight basipetal recoloration in 2–3 minutes.

If the reaction is allowed to continue in ovaries with normal reaction gradient until color becomes deep apically, the reduction gradient may be reversed in direction, or the young follicles of the apical region may not reduce at all, evidently because the intracellular indophenol has become toxic in the regions where it is greatest in amount. With still further progress of reaction, reduction may be completely inhibited. In ovaries with reversed reaction gradient, i.e., with acropetal progress of reaction, the reduction gradient may also be acropetal in progress; but in some cases observed it has remained basipetal. In short, the reduction pattern, like the reaction pattern, may be altered by various factors which affect the physiological condition of the ovaries; but what factors are concerned in determining direction of progress of reduction in a particular ovary with reversed reaction gradient remains uncertain. The experimental data in general suggest that not only the differential effect of inadequate nutrition and of high concentrations of α-naphthol but also the rapidity of indophenol reaction, length of isolation and of reaction period preceding reduction, differential intracellular accumulation of indophenol blue, and rapidity of decrease of oxygen content in the sealed preparation may influence direction and progress of reduction. There is evidently a gradual change in physiological condition following isolation, and the relation between physiological condition and experimental or incidental factors in a particular ovary or follicle string at a given time is not known. In ovaries with reversed reaction pattern physiological condition has been altered before reduction begins. The reduction pattern perhaps indicates the degree of alteration, and comparative experiments throw some light on factors chiefly concerned in some cases. However, since data concerning the normal reduction pattern are clear and consistent, it seems unnecessary to discuss at greater length the data of reduction patterns in these altered ovaries, since they are, at best, of minor significance.

On exposure to air of the solution containing the ovaries after reduction, reoxidation and recoloration of indophenol blue take place. With very low concentrations of indophenol reagents the color after reoxidation is usually less deep than before reduction, again suggesting that the living cells have broken down some of the very small amount of indophenol blue present. With higher concentrations of reagents depth of color following reoxidation is usually deeper than before reduction, evidently because the reagents or reduced indophenol blue continue to accumulate in the cells during the reduction period. The direction of progress of recoloration in reoxidation may be either acropetal or basipetal, according to the physiological condition of the ovaries and experimental conditions—for example, concentrations and possible toxic effects of reagents or of intracellular indophenol, length of period in low oxygen, and length of isolation period. In work with indophenol on various other organisms it has been found that regions which react most rapidly and deeply show reoxidative recoloration following reduction least rapidly, provided they are still in good condition. This suggests that they

are in general regions of greater oxygen demand than less rapidly reoxidizing regions. Similarly, in ovaries most nearly in normal condition—that is, with relatively low concentrations of reagents, reduction before intracellular indophenol becomes highly toxic, short period in low oxygen before reoxidation, and short isolation period—reoxidative recoloration progresses acropetally. But very often the highly susceptible young follicles of the apical region are injured before reoxidation can occur, and under these conditions the progress of recoloration may become partially or wholly basipetal; that is, apical regions are more inhibited or depressed than other parts. In Series 3 and 5 (pp. 155 ff.) recoloration progresses basipetally. The factor concerned in these cases is almost certainly the greater susceptibility of the more apical regions to low oxygen. With rapid increase of oxygen content reoxidation occurs almost at once or in a few seconds; with mere exposure of the solution to air it may be complete in a minute or two.

By addition of minute quantities of sodium hydrosulphite to the solutions containing ovaries which have already reacted, reduction may be brought about as rapidly as desired in open preparations. Oxidation of the hydrosulphite removes free oxygen from the solution. With even a small fraction of a milligram of hydrosulphite in 2–3 ml. of solution ovarian reduction may be almost instantaneous. With smaller amounts of hydrosulphite basipetal reduction occurs in open solutions of 2–3 ml. volume, the rate decreasing with decrease in concentration of hydrosulphite; and in sufficiently low concentrations, oxidative recoloration may follow within 2–5 minutes, or, with agitation of the solution to increase oxygen content of the solution more rapidly, it may occur in less than a minute. With this procedure the period in low oxygen is short and recoloration progresses acropetally unless other factors have produced differential toxic effects; as in other cases, more or less complete reversal of both reduction and reoxidation gradient results from these effects. A second addition of hydrosulphite after reoxidation brings about a second reduction; and this is followed, in turn, by a second reoxidation in solutions open to the air.

In still lower concentrations hydrosulphite serves to decrease oxygen content of the solution somewhat without bringing about reduction in the ovaries. If used in this way immediately before sealing, reduction in the sealed preparation occurs more rapidly than without hydrosulphite because oxygen content is already decreased at the time of sealing.

Differences in reduction and reoxidation with hydrosulphite in ovaries floating and in those a few millimeters below the surface are of interest. The small ovaries in early developmental stages are usually kept afloat by air in the tracheal system, while more advanced, heavier ovaries sink. After the indophenol reaction in a lot containing both floating and heavier ovaries a few tiny grains of hydrosulphite are dissolved with brief stirring in the 2–3 ml. of solution. The purpose of the experiment is to decrease oxygen content almost to the critical level for reduction, but not sufficiently to bring about reduction directly and immediately by hydrosulphite. With repeated trials the amount of hydrosulphite to be added can be estimated rather closely. With the proper amount, ovaries at the bottom, some 5 or 6 mm. below the surface, gradually reduce basipetally in 2–3 minutes. The ovaries floating just below

the surface show the beginning of reduction in the apical region, but it does not progress far and is followed in 2–3 minutes by reoxidation. However, if a floating ovary is pushed to the bottom while oxygen content is still low, reduction continues basipetally until complete; and, when it is allowed to rise to the surface again, reoxidation follows. Without agitation of the solution reoxidation in ovaries at the bottom gradually occurs as oxygen content increases after complete oxidation of hydrosulphite.

Late-oöcyte stages with chorion and the full-grown eggs react so much more slowly than the earlier follicles that their reduction is best observed independently of earlier stages. In what appears to be the normal pattern of reduction, decoloration in low oxygen progresses from the anteroventral tip posteriorly and in many eggs with a slight ventrodorsal differential below the anterior tip. Figure 6 will serve to indicate this pattern as well as the reaction pattern. Like the reaction pattern, this course of reduction is not evident in all eggs. In some a second reduction gradient extends from the posterior end anteriorly (see p. 148); and often, particularly if the reaction has been long continued, or the concentrations of reagents high, the ventrodorsal differential is not evident or appears only at the anterior tip. Data on two lots of eggs with different concentrations of reagents follow. Times given are from addition of indophenol reagents.

1. Half the eggs from ten mature ovaries in diamine 1/320,000, α-naphthol 1/400,000.

35 minutes: early reaction stages, anteroventral in almost all; a few with second reaction gradient posteriorly. Now sealed in small cell with additional tissue for rapid reduction.

50 minutes: reduction so rapid that earlier stages not observed, but posterior progress evident in some; others completely reduced. Reaction had not progressed beyond earlier stages before reduction in any. Now opened and a few drops of original reagent solution added. Recoloration took place in 1–2 minutes with deeper color and further progress of reaction than before reduction.

55 minutes: sealed again.

1–1¼ hours: reduction progressing posteriorly, except that extreme anteroventral tip now somewhat retarded in about 50 per cent, but in almost all this retarded region reduced completely before the posterior end. The ventrodorsal differential was not distinct, and in a few eggs the dorsal side appeared to reduce slightly earlier than the ventral. The retardation of reduction in the anteroventral region and the absence of the ventrodorsal gradient suggest differential toxic effect of the reagents or indophenol blue. At 1¼ hours reduction was complete or far advanced except in a few deeply colored eggs with no evidence of reduction.

1⅓ hours: opened; recoloration occurred in 1–2 minutes with anteroposterior and ventrodorsal gradient except in a small number with recoloration progressing from both ends.

1½ hours: sealed a third time.

1¾ hours: reduction as before, except that anteroventral retardation more frequent and extending farther posteriorly.

2 hours: reduction complete except in a few with anteroventral region not reduced and a few others with no reduction.

In this lot the successive reductions indicate a differential decrease in ability to reduce indophenol blue, progressing from the anteroventral tip posteriorly.

2. The other half of the eggs from the same ten ovaries as the preceding lot in diamine 1/640,000, α-naphthol 1/800,000, with concentrations half those of the other lot.

1½ hours: early to medium reaction with normal gradient pattern characteristic. Sealed with crowding in small cell; reduction evident in 5 minutes.

1¾ hours: reduction progressing posteriorly in almost every egg; ventrodorsal differential evident in most eggs lying on side. A few eggs show slight retardation in anteroventral tip.

Essentially similar reductions have been observed in many other eggs and advanced oöcytes. The further the reac-

tion is allowed to progress, the more frequently is reduction retarded and the greater the extent of the retarded region from the anteroventral tip. With extreme reaction and very deep color there is no evidence of reduction.

The eggs are so nearly opaque that it has not been possible to determine whether reaction and reduction gradients are primarily or only cortical or extend more or less throughout the egg. Also, the gradient pattern in the full-grown egg is evidently slight and readily altered or obliterated by differential toxic action of the reagents or of indophenol blue.

DISCUSSION

As regards what appears to be the normal pattern of indophenol reaction and reduction, the data are sufficiently clear and consistent to make discussion unnecessary. They are in accord with the data on differential reduction of methylene blue and Janus green (Child, 1942). Evidence as regards the factors concerned in some of the alterations of pattern—e.g., the longitudinal gradient reversals by high concentrations of α-naphthol and KCN—also appears clear and consistent; but, as regards some other alterations, it is uncertain which of the possible factors are concerned in particular cases (p. 156). However, there can be no doubt that the patterns of indophenol reaction and reduction and their experimental and other alterations are expressions of a physiological pattern and the effects of different conditions on it.

Whether activity of living cells is concerned in reoxidation or only presence of oxygen is necessary, reoxidation shows a gradient pattern, evidently related in some way to physiological pattern. It seems possible that, with gradual increase in oxygen content of the medium, differences in oxygen demand associated with differences in physiological condition at different levels may, at least under some conditions, influence rate of reoxidation of indophenol blue; probably here, also, as in the primary reaction, differential activity of an oxidase is concerned. Whatever the factors concerned in differential reoxidation, the relation of indophenol blue to physiological ovarian pattern appears in at least three aspects: the differential indophenol reaction, the differential reduction of intracellular indophenol blue, and the differential intracellular reoxidation of reduced indophenol blue.

Moreover, young ovaries and the earlier developmental stages in the apical regions of older ovaries and of isolated follicle strings show a gradient of partial or sometimes complete decoloration, or apparent retardation of reaction, decreasing basipetally, in very low concentrations of indophenol reagents, even while reaction is progressing in regions farther basal, and without decrease of oxygen content of the medium. A similar decoloration gradient was observed in low concentrations of methylene blue and Janus green (Child, 1942). As pointed out in this and the earlier paper, these decoloration gradients suggest that the earlier developmental stages are able to break down irreversibly in some way small amounts of methylene blue, Janus green, and indophenol blue and that this ability decreases and disappears basipetally with progress of follicle development. If this is the case, the basipetal decrease of this ability constitutes still another aspect of the relation of indophenol blue to physiological pattern of the ovaries.

The virtual coincidence of the longitudinal axis of the ovary and its longitudinal gradient pattern with the longitudinal axis of the insect body suggests that direction of the ovarian axis originates in relation to the longitudinal body pattern. The longitudinal gradient characteristics of pattern in the developing ovary evidently result from difference in physiological condition associated with progress of development in the follicle strings and follicles in the axial direction. The ventrodorsal component of pattern apparently originates in the difference in condition between the outer ovarian surface and other parts. The gradient pattern in oöcyte and full-grown egg apparently originates as a part of the ovarian pattern.

SUMMARY

1. With the use of very low concentrations of dimethyl-paraphenyldiamine (para-aminodimethylaniline) and α-naphthol in a modified Ringer solution it is possible to obtain the indophenol reaction in isolated living ovaries, single follicle strings, single follicles, oöcytes, and full-grown eggs of *D. hydei*.

2. The reaction pattern regarded as "normal" is that observed in the earliest imaginal stages and in rapidly developing ovaries, follicles, and later stages from well-fed flies, with the lower concentrations of reagents found by experiment to be usable with living material. Its most conspicuous feature is a gradient consisting in progress basipetally or posteriorly of intracellular appearance and accumulation of indophenol blue in intact living ovaries of all stages from emergence of flies, in single follicle strings, single follicles above a certain size, late oöcytes, and full-grown eggs. There is also evidence in many follicles above a certain size and in later stages of a slight indophenol reaction gradient decreasing from the ventral side dorsally, i.e., from the outer surface of the ovary inward; but this gradient is not always visible.

3. The longitudinal reaction gradient is reversed in direction in ovaries with development inhibited by inadequate nutrition or starvation and by differential susceptibility to concentrations of α-naphthol above a certain limit; and reaction is retarded and may be partly reversed in direction by high concentrations of diamine. This reaction gradient is also reversed or obliterated by differential susceptibility to a certain range of concentrations of KCN.

4. Ovaries left in Ringer solution until evidently dead, or killed in boiling water, distilled water, KCN M/500–M/2,000, or $HgCl_2$, show completely uniform indophenol reaction with depth of color depending upon concentration of reagents.

5. With decrease of oxygen content of the medium, intracellular indophenol blue is reduced to a colorless form by living ovaries in the presence of the indophenol reagents, and reoxidation with return of color occurs when oxygen content is again sufficiently increased. Both reduction and reoxidation show gradient pattern. The reduction pattern regarded as "normal" is a gradient in the same direction as the reaction, but in ovaries with the reaction pattern altered or reversed by experimental or other conditions reduction and reoxidation patterns differ according to conditions. After extreme reaction reduction does not occur. The reoxidation gradient regarded as normal is acropetal but is also altered or reversed by differential toxic effects.

6. Rapid decrease of the oxygen content of the medium and reduction of in-

tracellular indophenol blue may be brought about in solutions open to air by the addition of minute amounts of sodium hydrosulphite. With complete oxidation of the hydrosulphite and diffusion of oxygen into the fluid reoxidation soon follows.

7. The data indicate that the longitudinal gradient pattern of the egg originates as a part of the longitudinal ovarian pattern and its ventrodorsal pattern in relation to the differential in condition between the outer surface of the ovary and other parts.

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THE RATE OF ELONGATION AND CONSTRICTION OF DIVIDING SEA-URCHIN EGGS AS A TEST OF A MATHEMATICAL THEORY OF CELL DIVISION[1]

(Two figures)

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WHILE many theories have been proposed to explain the phenomena of cell division (Wilson, 1934), only recently has a theory of cell division been derived in a mathematical form which is capable of quantitative prediction. This theory, developed by Landahl (1942*a*, *b*), Rashevsky (1939*a*, *b;* 1941), and Young (1939*a*, *b*), predicts in a manner capable of experimental verification or refutation, relations between the metabolism of a cell and the process of division, the upper limits of the size of a cell, the dependence of cell division upon certain external conditions, and describes, again mathematically, the gross changes of the cell during division.

This theory is derived from the following considerations. The metabolizing cell consumes substances coming either from the external medium or from the cytoplasm and produces other substances. The consumption and production of these substances set up differences in their concentration, and diffusion of the metabolites follows. In diffusing they exert forces on the cell by an unequal molecular bombardment and by a viscous drag. These forces tend to deform the cell, while the surface forces in the membrane oppose any changes from the spherical shape. If the diffusion forces are greater than the surface tension forces, the cell becomes unstable and elongates. Elongation is followed by cell division.

For a quantitative derivation a more clearly defined picture is needed. A cell may be regarded as a sac containing a plastic-like material in which there are colloidal particles (Rashevsky, 1938, 1940) and to which are attached respiratory and other catalysts. If the distribution of the particles is uniform and their number great, the concentration gradients of the metabolites may be calculated exactly for very simply shaped cells (e.g., spherical or ellipsoidal) from well-known mathematical equations, or approximately for cells of any shape by an approximation method (Rashevsky, 1940). From the gradients the mean relative rate of elongation can be determined by a transformation of Betti's equation (Young, 1939*a*), using transformations suggested by Goodier (1936) and Nadai (1928, 1937). These transformations make the equations of elasticity apply to plastic bodies so that strain in an elastic body corresponds to the rate of strain in a plastic body (Young, 1939*a*).

As the cell elongates, the surface forces opposing the elongation increase until they balance the volume forces, and this results in a cessation of the average elongation. Then the average rate of elongation for the cell as a whole be-

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comes zero, but the cell material near the equator of the cell continues to be displaced outward, i.e., there is an elongation of the equatorial portion. But the elongation at the equator demands a corresponding constriction of the same region. The rate of constriction at the equator may then be computed (Rashevsky, 1941; Young, 1939*b*).

A relation may also be obtained that expresses the size of a cell at which a finite elongation sets in, in terms of the metabolic rate and certain physical constants of the cell (Rashevsky, 1938).

Thus the theory leads to an expression for the upper limit to cell size as a function of metabolism (Rashevsky, 1938) and two equations—one containing the rate of elongation of the cell (Rashevsky, 1939*a*; see also Landahl, 1942*b*) and, therefore, expressing the length of the dividing cell as a function of time, the metabolism (type and rate), the surface tension at the membrane, and certain physical constants, known or determinable in principle; and the other defining the rate of constriction as a function of the same quantities. For the derivation of these equations the reader is referred to Rashevsky (1940), and Landahl (1924*b*). The elongation equation is, in integrated form:

$$\epsilon = \frac{A}{B + A\,e^{-A(t-t_0)}}, \qquad (1)$$

where

$$\epsilon = \frac{r_1 - r_0}{r_0},$$

where t is the time, t_0 is determined by the choice of the zero point of the time, r_0 is the initial radius of the cell, $2r_1$ is the length on the axis of elongation, and $2r_2$ is the width computed from length and volume.

This equation contains two constants, A and B, and is rather simple in form. The theoretical derivation of this equation involves a rather detailed consideration of complex physical processes, and for the details the reader must be referred to the extensive mathematico-biophysical literature.

The constants A and B are rather complex in their structure, being determined through a number of physicochemical constants of the cell. The actual expressions are as follows:

$$A = \frac{1}{r_0}\left[\frac{K}{1+b} - \frac{3\gamma}{4\eta}\right],$$

$$B = \frac{1}{4r_0}\left[\frac{K}{(1+b)^2}(1-3b) + \frac{3\gamma}{4\eta}\right],$$

$$K = \frac{RT\mu q r_0^3}{4M\eta D_e},$$

R = gas constant,

T = absolute temperature,

μ = a constant determined by the molecular structure of the cytoplasm (see Rashevsky, 1940, p. 39),

η = viscosity of cytoplasm (see Heilbrunn, 1928),

γ = surface tension, i.e., surface force (see Cole, 1932),

D_e = effective external diffusion coefficient (*Intl. Critical Tables*, Vol. 5),

$b = \frac{2D_i}{D_e}$, where D_i is the internal diffusion coefficient (D_i is less than D_e, its value not being at all critical).

The parameters M and q are the so-called "effective molecular weight of metabolites" and "effective metabolic rate." For the definition and discussion of these quantities the reader is referred to the literature in mathematical biophysics (Rashevsky, 1940).

If all the above-listed physical constants of the cell could be determined separately, i.e., (1) the physical conditions of the experiment (temperature, volume of the dividing cell), (2) the physical constants (gas constant, internal

and external diffusion coefficients of the metabolites), and (3) sufficiently accurate estimates of the physiological constants (viscosity of cytoplasm, surface forces, and metabolic rates [for values of the last see Needham, 1931]), we would have the knowledge of the constants A and B and thus could predict not only the shape of the elongation curve but also its absolute size. This is still a rather remote goal, and in the present paper we propose to restrict ourselves to a more limited problem, namely, of checking whether the elongation of an egg is represented by an equation of the type (1), thereby determining the constants A and B. These values must be plausible in view of the possible values of the basic parameters defined above. This turns out to be the case (Landahl, 1942*b*).

Examination of equation (1) shows that the elongation curve is S-shaped, the position of the inflection point and the value of the maximum slope determining the constants A and B. One might think that an S-shaped curve can be represented by many other types of equations and that therefore the theory does really not predict anything beyond the statement that the elongation curve is S-shaped. This, however, is not so, for not every S-shaped curve can be represented by equation (1), not even approximately. To give an example from physics, we may refer to the curve which gives the relation between the absolute temperature and the specific heat of a solid. This is also S-shaped. But nothing simpler than the well-known Debye equation can represent this curve adequately.

Thus the theory not only states that the elongation curve is S-shaped but also states that it can be represented by equation (1). We shall see that this prediction is verified experimentally.

A further verification is obtained by considering the equation for the constriction of a cell, which is of the form

$$r^2 = r'^2 + 2Qt\,, \qquad (2)$$

in which $2r$ is the diameter of the neck at time t, $2r'$ is the diameter of the neck at $t=0$, t is time and Q is another constant related to the constant K by the equation

$$K = \frac{88Q}{r_0}. \qquad (3)$$

The theory which leads to equation (2) shows that it will hold only within a limited range of values of r. It is not expected to hold for the early stages and for the very latest stages of constriction. It is, however, of interest that the constant Q is related in a simple manner to A and B. Thus, if we compute A and B from the comparison of equation (1) with experimental data, we should be able to estimate the order of magnitude of Q. This actually turns out to be the case. Landahl (1942*b*) considers a quantity K, the same as mentioned above, which can be derived approximately either from A and B, by means of the equation

$$K = \frac{r_0(A + 4B)}{2} \qquad (4)$$

or from Q by means of equation (3). According to Landahl, the value of K, computed from the constriction data by equation (3), should be of the *same order of magnitude* as that computed from the elongation data by equation (4). Table 1 shows the comparison, for different eggs, as described later. Each value is an average obtained from a number of eggs.

It is in the nature of the derivation of these equations that their results be averages, containing no analysis of the movement of the cell material by parts; and, in fact, it is independent of them. Thus, movements in the mitotic spindle do not change the conclusions in any way. The specific metabolic chain is again of relatively lesser importance, since only a

few of the metabolites produce appreciable forces, and the expression for these forces may be given in terms of an effective rate—positive when the forces are directed outward, negative when they are directed inward. In the derivation, only average concentrations of metabolites on the different surface elements of the cell are considered, but the result must be physically the same when a more exact distribution of concentrations is used (Young, 1939*a*). Surface tension as used in the derivation is really a combination of the elastic properties of the membrane itself and the interfacial tensions at the membrane; but these vary in the same way, so that an effective surface tension may be used as a first approximation. To justify these procedures of approximation and neglect of exact structure, it is noted that, while no two *Arbacia* eggs are identical in fine structure, they all divide in essentially the same way; and it is assumed, therefore, that the variations in fine structure are of no great importance in determining the gross features of cell division (genetic variation excepted). It has been shown (Williamson, 1939, 1941; Householder and Williamson, 1942) that electrical forces may be considered as negligible, as far as their effects on the gross elongation or constriction are concerned.

TABLE 1

TABLE OF PARAMETERS

Parameters	Normal with Membranes	Shaken	Treated with KCl	Treated with KCN	Second Cleavage Shaken
A sec^{-1}	0.025	0.024	0.020	0.020	0.025
B sec^{-1}	0.22	0.059	0.031	0.048	0.071
$Q10^{-8}$ cm^2 sec^{-1}	2.6	2.9	3.9	2.8	1.5
K from eq. (4) in 10^{-3} cm sec^{-1}	1.7	0.45	0.26	0.36	0.45
K from eq. (3) in 10^{-3} cm sec^{-1}	0.62	0.73	1.00	0.72	0.48

Published data for a completely suitable comparison of the theory with experiment were not available. Churney (1936) had made direct measurements by eye with a micrometer microscope on the elongation of *Arbacia* eggs. But these data were not accurate enough for the early stages of the elongation, because of difficulties in determining the axis of elongation; and the data did not include measurements of the rate of constriction. Chambers produced a moving-picture film which, among other things, contains pictures of cleavage of the sea-urchin egg. From this film enlargements were made and measurements taken. While the general shape of the curves was that predicted, it was difficult to be sure about certain phenomena, because the stop-motion pictures were taken at irregular intervals, which were not accurately known. In addition, the cells were sometimes too close to other cells, so that their external diffusion fields possibly interfered; and, finally, the pictures were made for educational purposes, and focus was on general detail rather than on a sharp membrane boundary, so that there was some difficulty in making measurements as precisely as required. In the absence of data obtained under conditions approaching those assumed in the theory, it was necessary to measure dividing cells under controlled conditions.

Sea-urchin eggs were used because so many physiological data are already available. Eggs were collected from a ripe female, washed in filtered sea water, and a thin suspension of sperms added. Since, in order to verify the theoretical conclusions, it was necessary for the cells to elongate freely while dividing and then to separate as much as possible, the restricting fertilization membranes were broken by various means. Shaking in a test tube, passing eggs through silk bolting cloth, or treating with Ca-free sea

TABLE 2*

TABLES OF MEASUREMENTS

4a, NORMAL, 23.8° C.

t	$2r_1$	$2r$
48.75	75.9	73.6
49.00	74.8	73.4
49.17	75.9	73.4
49.32	74.8	73.3
49.50	75.9	73.1
49.67	75.6	73.1
49.83	75.9	72.2
50.00	75.9	71.7
50.17	76.5	71.4
50.33	77.6	70.3
50.67	78.8	68.6
50.83	79.0	67.4
51.00	79.6	66.9
51.17	80.7	64.3
51.33	81.6	62.3
51.50	81.9	60.6
51.67	81.9	57.8
51.83	82.2	56.1
52.00	82.7	53.3
52.17	83.0	51.0
52.33	83.3	48.7
52.92	84.4	43.9
53.25	84.4	41.1
53.50	83.9	39.7
53.75	83.9	.....

11a, SHAKEN, 24.5° C.

42.50	76.5	73.7
43.00	76.5	73.7
43.25	76.2	73.1
43.50	75.9	72.5
43.75	77.0	71.7
44.00	78.8	70.8
44.25	79.3	70.0
44.50	79.8	68.0
44.75	82.1	68.3
45.00	83.8	63.2
45.25	86.6	60.0
45.50	88.4	56.1
45.75	92.4	50.7
46.00	95.1	45.3
46.25	97.6	40.0
46.50	100.0	34.0
46.75	102.6	28.3
47.00	103.3	23.2
47.25	104.5	18.7
47.50	105.8	13
47.75	106.6	11
48.00	107.0	.....
48.25	107.0	.....
49.00	106.8	.....
49.75	105.0	.....
51.50	101.6	.....
52.50	101.0	.....
56.25	98.0	.....
60.25	94.9	.....
62.83	96.2	.....

12a2, SECOND CLEAVAGE (11a), 24.5° C.

t	$2r_1$	$2r$
68.83	58.7	47.5
69.50	58.8	47.5
69.83	58.8	48.0
70.17	61.0	46.9
70.33	61.4	45.8
70.50	62.0	45.1
70.75	64.1	43.9
71.00	64.7	42.3
71.25	66.9	39.9
71.50	69.0	35.8
71.75	71.2	32.4
72.00	73.3	28.3
72.25	74.1	24.2
72.50	76.0	20.2
72.75	77.6	16
73.00	78.0	11
73.25	78.3	.....
73.50	80.0	.....
73.75	80.0	.....
74.83	79.5	.....
76.50	77.6	.....
78.50	76.3	.....
80.50	75.5	.....

16a1, SECOND CLEAVAGE, 24° C.

62.75	58.8	44.5
68.50	59.3	45.8
69.50	60.4	45.8
70.00	62.5	45.8
70.33	64.7	43.1
70.67	67.4	40.4
71.00	70.6	37.2
71.33	72.8	32.3
71.67	74.9	27.5
72.00	75.5	22.9
72.33	77.1	18.9
72.67	77.6	13.5
73.00	78.1	9
73.50	78.2	.....

25a1, SHAKEN, 24.5° C.

50.00	69.4	69.3
51.00	69.6	69.3
51.50	69.1	68.8
52.00	69.1	68.5
52.50	69.4	67.9
53.00	69.6	66.8
53.50	70.5	65.7
54.00	72.1	64.6
54.50	73.5	62.9
55.00	74.9	60.7
55.50	76.3	58.5
56.00	77.7	56.3
56.50	79.1	53.2

*The serial number is followed by a designation of the treatment, i.e., normal—untreated, shaken—fertilization membrane removed by shaking, KCN—removal by shaking and addition of KCN, KCl—removal by treatment with KCl. The temperature is given last. Under the column headed t are given the times from fertilization in minutes. In columns $2r_1$ and $2r$ are given respectively, the lengths from pole to pole and the widths at the equator, measured in microns.

TABLE 2—*Continued*

25a21, SHAKEN, 24.5° C.—*Continued*

t	$2r_1$	$2r$
57.00	80.2	50.4
57.50	81.3	47.3
58.00	83.0	45.1
58.50	84.4	41.8
59.00	85.8	39.5
59.50	86.3	36.8
60.00	86.6	34.0
60.50	88.6	30.6
62.00	89.1	26.7

25a23, SHAKEN, 24.5° C.

49.25	71.8	66.3
50.00	73.5	61.3
50.50	76.6	57.9
51.00	79.9	52.6
51.50	83.6	45.1
52.00	88.0	38.4
52.50	89.7	30.1
53.00	91.4	20
53.50	92.2	12
55.00	92.6	.....
56.00	92.2	.....

32a, KCL, 24.8° C.

37.00	73.2	73.2
40.00	73.2	72.7
43.25	73.5	72.7
44.00	75.0	71.6
44.75	76.8	66.8
45.25	80.3	63.4
45.75	84.7	56.8
46.50	93.4	40.7
47.00	98.6	26.2
47.50	101.1	11
47.90	103.5	.....
48.50	107.9	.....
49.50	114.0	.....
50.50	115.3	.....
51.00	114.7	.....
53.00	112.5	.....

32b, KCL, 24.8° C.

39.00	70.5	69.1
40.58	73.2	66.1

32b, KCL, 24.8° C.—*Continued*

t	$2r_1$	$2r$
41.00	74.5	62.8
41.50	78.1	56.3
42.00	84.7	45.9
42.50	91.5	32.8
42.75	96.2	15.3
43.00	101.5	7
43.50	106.6	.....
44.00	114.8	.....
45.00	117.5	.....
45.83	118.8	.....
46.50	118.8	.....
47.08	118.8	.....
47.42	118.0	.....
47.75	118.0	.....
48.17	118.8	.....

41a, 10^{-4} M KCN, 24.0° C.

60.00	69.9	68.3
61.25	70.8	68.0
62.00	72.7	64.2
64.00	84.7	46.4
64.75	91.0	31.4
65.50	95.1	17.2
66.25	97.0	10
67.00	98.6	8
69.00	100.5	.....

45c18, 5×10^{-5} M KCN, 22.7° C.

52.25	66.9	69.9
54.50	66.9	69.9
55.25	66.9	69.9
56.00	67.2	69.9
56.75	68.3	69.6
57.50	69.4	68.0
58.25	72.0	65.1
59.50	79.6	54.0
60.75	90.3	34.7
61.75	95.2	13
62.75	96.8	.....
64.25	96.5	.....
65.00	96.2	.....
66.50	94.1	.....
68.50	93.5	.....
69.75	92.5	.....
70.75	91.1	.....
71.50	89.2	.....

water or isosmotic KCl were the methods used. Although different percentages of membranes were removed by different techniques, for the purpose of these data the mode of treatment did not seem to matter. Eggs were treated for removal about 1.5 minutes after insemination. Shortly before first cleavage was due, some of the eggs were placed on a slide and covered with a cover glass supported

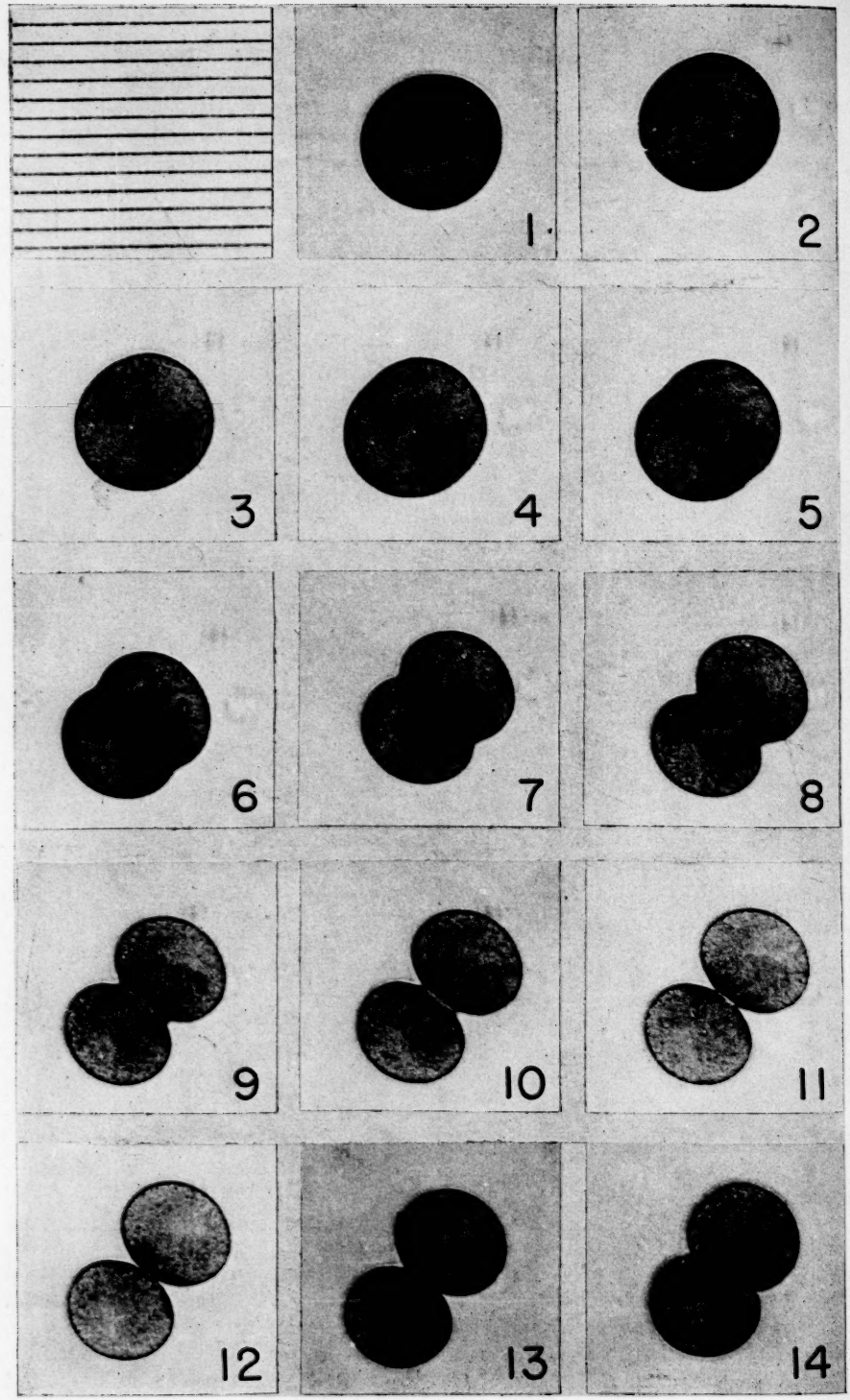

FIG. 1

to avoid pressure upon the eggs. A number of eggs were left with membranes. Eggs were photographed at exactly known times. One observer would count seconds and record time of exposure, while the other released the shutter. Temperatures were recorded.

Sixty-two series were made in all; each consisted of about 10–25 exposures for each dividing egg. Enlargements were made, and the images measured on the photographs. Absolute measurements were known because a slide micrometer was photographed at the same time and a corresponding enlargement of it used for determining the magnification. The data include measurements of eggs dividing in various concentrations of KCN in sea water.

The measurements of representative samples of various groups are given in Table 2. The last digit of each of the measurements of lengths $2r'$ and the width of the constricting region $2r$ are not much more than rough estimates. The measurements from one egg (25a1), which is atypical in respect to its constriction curve, are given. Five eggs which definitely showed this pattern were from the same batch of 8 eggs. One of these was definitely not of this type. This type of constriction curve is not covered by the theory. The measurement of the width of the neck is difficult to obtain when constriction is well advanced. The measurements were thus discontinued when the uncertainty warranted it.

Figure 1 shows a representative series of photographs of an egg from which the fertilization membrane had been broken. The exact time of each exposure is given in the legend. (Twenty-nine photographs of this dividing egg were made; only alternate photographs are reproduced here.)

When these data, in terms of time (abscissas) and total length (ordinates) are plotted and superimposed upon the theoretical curve described, the degree of agreement can be seen (from Fig. 2) to be remarkable. It is to be emphasized that the curve is not merely an empirical fit of the data but is rationally derived. That an empirical curve should be capable of being drawn through a string of points is not very significant, but it is significant that the mathematical biophysicist should be able to set up an equation yielding a curve whose parameters are not all arbitrary but mean something in terms of physiology, physics, and chemistry.

Other mathematical expressions have been derived by Landahl (1942*b*) which have been checked against observations by the use of the above-mentioned data. Those expressions include the relation between the constants Q and r_0 and a relation between the rate of elongation of a cell and the elongation expressed in special units. This enables an interesting comparison of theory and experiment. For the discussion of those questions we refer to Landahl's paper (1942*b*).

It is noteworthy to biologists that these equations do not depend upon specific cell contents, such as the nucleus or spindle; and, while it is not concluded that the nucleus and spindle play no role in cell division, it is probable that these cellular organs do not directly affect the gross mechanism of cell division. That

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FIG. 1.—Representative series of photomicrographs of a dividing sea-urchin egg from which the fertilization membrane had been almost completely removed (11a of Table 2 and Fig. 1). Twenty-nine photographs of this dividing egg were made; only alternate photographs are reproduced here. The first picture shows scale of magnification: one scale division = 10μ. The exact time after fertilization of each exposure is as follows: *1*, 42.08 min.; *2*, 43.00 min.; *3*, 43.50 min.; *4*, 44.00 min.; *5*, 44.50 min.; *6*, 45.00 min.; *7*, 45.50 min.; *8*, 46.00 min.; *9*, 46.50 min.; *10*, 47.00 min.; *11*, 47.50 min.; *12*, 48.00 min.; *13*, 49.00 min; *14*, 51.50 min. Temperature, 24.5° C.

this is likely has been common knowledge with respect to nonnucleated blue-green algae, which divide without benefit of a discrete nucleus, and the enucleated sea-urchin egg fragments, which may also divide (Harvey, 1936).

It happened that the theoretical considerations went only to the maximum elongation of the dividing blastomeres or to the point marked *A* on the curve in Figure 2. The data clearly show that after this time the two blastomeres come together, thus slightly reducing the apparent total length of the two cells.

The data describing the constriction between the two blastomeres show fair agreement through most of the range. The discrepancy looks worse than it really is, for two reasons. First, there is the fact that, while the theory assumes that the two cells separate completely, actually they do divide and form separate membranes; but they stick together, so that an end-point is difficult to determine from the pictures. And, second, the theory referred to here does not deal with the very early stages of constriction.

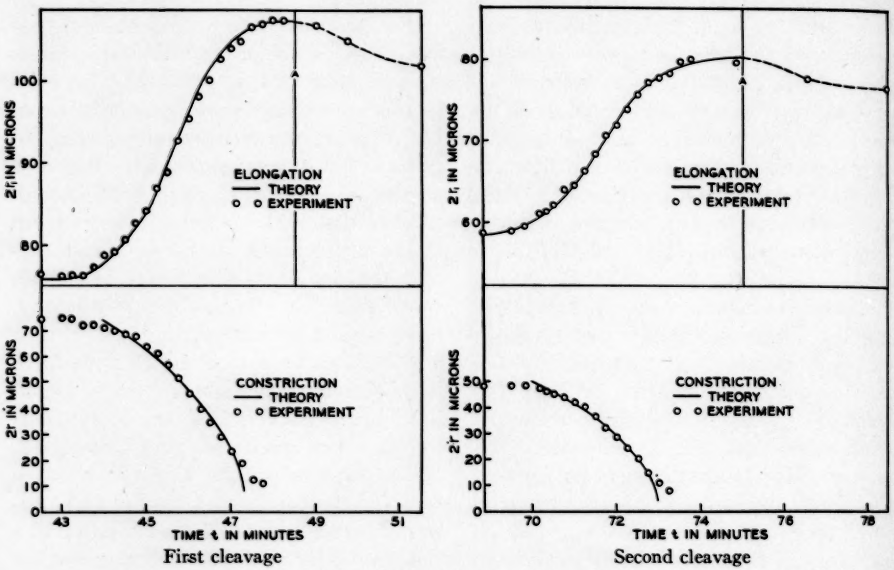

FIG. 2.—Elongation and constriction curves for dividing sea-urchin eggs compared with theory: *left*, first cleavage (11a of Table 2 and Fig. 1); *right*, second cleavage (one of the daughter cells of 11a). The experimental values were obtained by measuring photomicrographs (lengths for elongation; widths for constriction) and plotting these values against time after fertilization. Theoretical values were obtained from equations (1) and (2), given in the text, using $A = 0.029$ sec^{-1}, $B = 0.069$ sec^{-1}, $t_0 = 2{,}766$ sec, $2r_0 = 75.5 \times 10^{-4}$ cm, $r'_0{}^2 = 1.91 \times 10^{-4}$ cm^2, and $Q = 3.36 \times 10^{-8}$ cm^2 sec^{-1} for the parent cell, and using $A = 0.025$ sec^{-1}, $B = 0.065$ sec^{-1}, $t_0 = 4{,}332$ sec, $2r_0 = 58 \times 10^{-4}$ cm, $r'_0{}^2 = 1.54 \times 10^{-4}$ cm^2, and $Q = 1.75 \times 10^{-8}$ cm^2 sec^{-1} for the daughter cell.

Figure 2 also shows the data and theoretical curve for the second cleavage; the measurements were taken on one of the daughter blastomeres of the egg represented in the same figure. Again, the agreement is sufficiently good. Similarly, this constriction curve is a good fit, with the explanation for the apparent discrepancy as explained above. The parameter Q is about one-half that for the whole cell, as is predicted from the theory.

These data will be kept on file in the Office of Mathematical Biophysics at the University of Chicago. Prints of the dividing eggs will be lent to any investigator for further analysis.

SUMMARY

1. Rational equations were derived by Rashevsky, Young, and Landahl from theoretical considerations that the consumption and production of certain substances set up differences in their concentrations and therefore differences in rates of diffusion flow. In diffusing, forces are set up which tend to deform the cell, while this tendency is opposed by the surface tension of the membrane. If the diffusion forces are greater, the cell may elongate. Further equations were produced which express the length and constriction of the dividing cell as a function of time. The equations do not depend upon cell contents, such as nucleus or spindle. It was the purpose of the work reported here to experimentally test these equations.

2. Dividing sea-urchin eggs (*Arbacia*), with fertilization membranes removed, were photographed at accurately known time intervals. Measurements of the enlarged photographic images were plotted against time, and the data superimposed on the theoretical curves with remarkable agreement.

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EFFECTS OF CERTAIN B VITAMINS ON POPULATIONS OF TETRAHYMENA GELÆII

(Seven figures)

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IN MUCH of the older literature on protozoan populations the conclusion that accumulated waste products are responsible for cessation of growth is commonly found. As a result of recent studies this conclusion must be modified. In several investigations (Taylor and Strickland, 1938; Johnson and Hardin, 1938; and Kidder and Stuart, 1939) where the bacterial food supply was controlled it has been demonstrated that excretion products accumulating in the medium for long periods of time do not affect the rate of reproduction.

Phelps (1936) in studies on *Glaucoma piriformis* grown in pure culture on Difco yeast extract and on yeast autolysate found that a population of this ciliate can grow to tremendous densities before the effect of excretory products upon the division rate is observed. Lwoff and Roukhelman (1929), using the same organism, found that growth stops long before the nutrient materials, as measured by total N, amino N, amide N, and peptone N are exhausted in the medium and concluded that cessation of growth is caused by the accumulation of waste products in the medium. However, the fact that other factors may be operating in such cultures was indicated recently in two reports. Hall and Shottenfeld (1941) in studies on populations of *G. piriformis* found that quite different maximal densities were obtained when they used two different preparations of Difco Tryptone as the culture medium. In one preparation they obtained a maximal density of about 150,000 organisms per cubic centimeter; in the second preparation the maximal density was about 65,000 organisms per cubic centimeter. By adding vitamin B_1 to the second preparation they could increase the maximal density in the cultures to about 110,000 per cubic centimeter, suggesting that the lower maximal density obtained originally with the second preparation might be due to a lower content of vitamin B_1 in that preparation. Johnson (1941) reported that, when small amounts of vitamin B_1 were added to cultures of *Tetrahymena geleii*, grown in sterile culture on yeast extract, which had ceased to increase in numbers, further increases occurred.

In this study the authors investigated further the effects of vitamin B_1 on the growth of populations of *T. geleii* and also made some observations on the effects of three other components of the B complex on such populations. One series of experiments was designed so that the course of such populations could be followed for a period of more than 3 months.

MATERIALS AND METHODS

The ciliate used in this investigation has been used as an experimental organism in this laboratory for several years. It was originally isolated in sterile culture by J. O. Thomas, and its morphol-

ogy and taxonomy have been worked out by Furgason[1] (1940).

Unlike many ciliates, *Tetrahymena* grows in the absence of other living organisms in most peptone and yeast-extract media. The basic medium in this study was the following solution: Difco proteose-peptone, 5.0 gm.; dextrose, 5.0 gm.; triple-distilled water, 1.0 liter. The initial pH of the medium was 6.8. Solutions of the vitamins were sterilized and added separately. The thiamin chloride used was obtained from Merck and Company; the riboflavin, vitamin B_6 hydrochloride, and para-aminobenzoic acid, from the S.M.A. Laboratories. Conditioned medium was prepared in the following way: *Tetrahymena* were grown in large flasks of the basic medium; they were killed by a brief exposure to a temperature of 50° C.; and, after the dead organisms settled to the bottom of the flask, the culture medium was removed with sterile pipettes.

In the beginning of the investigation the experiments were set up in Erlenmeyer flasks, and these were sampled from time to time for population counts. However, the fact that a single contamination from sampling ruined the whole experiment led to the use of test tubes. An amount of basic medium at least 50 per cent larger than necessary for an experiment was prepared in a large flask and inoculated with the ciliates. The flask was then placed in a specially made rack that held it about 30° above the horizontal and opened in a Bunsen flame. Ten-cubic-centimeter portions were then removed with sterile pipettes and placed in 18×150-mm. Pyrex test tubes. The flask was thoroughly shaken after each removal, and samples were killed at regular intervals for counts of initial population. The test tubes were then arranged into control and experimental series in a regular manner, so that tube 1 in the control series was taken immediately before tube 1 in the experimental series, tube 2 in the control before tube 2 in the experimental, etc. Each series in all of the experiments consisted of twelve tubes. In some of the experiments a few tubes were kept for longer observation. All experiments were started from stock cultures which were growing in the basic medium and which were in the logarithmic phase of growth. All cultures were maintained in a constant temperature room at 25° C.

Counts were made by a uniform Sedgwick-Rafter technique essentially like that described by Hall, Johnson, and Loefer (1935). As used in this study, the method is as follows: A given sample of organisms (usually 1 cc.) is diluted appropriately with 10 per cent formalin solution in a test tube. After this dilution with preserving fluid the sample may, if necessary, be set aside for future counting. When ready to count, the contents of the tube are shaken thoroughly and poured into a 50-cc. beaker. A standard Sedgewick-Rafter counting cell (1 mm. deep) is put in position on a microscope, and the cover slide of the cell is placed so that small openings remain at three corners. The sample is well mixed; and an aliquot of it, amounting to slightly over 1 cc., is taken up in a medicine dropper and introduced into the counting cell. This is run into the cell until small drops of fluid stand up at the three corners, but care is taken not to float the cover slide. (It is easy to avoid floating if an "extra-thin" microscope slide is used as a cover instead of the cover that is usually furnished with the cell.) The cover slide is

[1] Furgason states that the ciliates which have been called *G. piriformis*, *Colpidium campylum*, and *C. striatum* are, in morphological characteristics, all like the species which he has designated *T. geleii*.

then twisted squarely on the cell, and the excess liquid wiped off. The organisms are permitted to settle at least 10 minutes. A count is taken of the number of organisms in each of 25 "squares" of the cell, using a standard Whipple ocular micrometer to determine the squares. Counts are made using the low power of a compound microscope. In all of the data presented, with the exception of the data on "total populations," the average of two counts from each sample is used. The results are then expressed in numbers of organisms per cubic centimeter of culture by the following formula:

$$\frac{\text{Average No. per square} \times \text{dilution}}{\text{Actual area of the ocular square}} \times 1{,}000.$$

Diluted formalin is kept on hand and is measured from a burette into test tubes. The samples are withdrawn from the cultures with aseptic precautions and added directly to the formalin. With experience, one is able to estimate the required dilution for any given culture. When a hand talley counter is used, any number up to 50 per square can be counted without undue loss of time.

Certain precautions are necessary if avoidable errors are not to be introduced into the method. (1) The culture must be well shaken before sampling. (2) As living organisms tend to cling to the sides of any small-bore pipette, the organisms must be sucked in and out of the pipette two or three times before the sample is taken up. Thus, the loss of organisms is out of the whole culture not out of the sample and is negligible. (3) The 25 squares are picked by following a regular pattern so that all parts of the cell are sampled. The number 25 was selected on the basis of preliminary tests which indicated that less than 20 or 25 might be inadequate and that more contributed little more to the accuracy. (4) The operator must not be looking through the microscope when moving the cell from one position to the next. When looking while selecting, the inevitable tendency is to pick locations containing larger numbers. (5) The cover slide should not be floated off the rim of the cell. Otherwise the organisms from a thickness of more than 1 mm. of liquid may be counted, even though the excess liquid is forced out upon closing the cell.

The following data will give some indication of the validity of the method used. In the early stages of the work this was tested by taking ten separate samples from a flask culture in quick succession. The following counts were made from ten samples taken from a 30-hour yeast autolysate culture: 78,125, 76,339, 74,553, 77,678, 76,786, 76,607, 75,446, 77,232, 75,893, and 75,000. Here the percentage standard deviation is 1.4 per cent. In the first experiment to be described, four separate samples, taken during the inoculation of the tubes at the start of the experiment, gave counts of 5,716, 5,794, 5,775, and 5,676, with a percentage standard deviation of 0.8 per cent. In the second experiment four similar samples gave counts of 4,714, 5,382, 5,127, and 5,068, with a percentage standard deviation of 4.5 per cent; in the third experiment similar samples gave counts of 9,036, 8,678, 8,786, and 9,186, with a percentage standard deviation of 2.2 per cent; in the fourth experiment similar samples gave counts of 11,678, 12,750, 12,178, and 12,178, with a percentage standard deviation of 3.1 per cent; and in the last experiment four samples from the culture used to inoculate the tubes gave counts of 4,321, 4,361, 4,184, and 4,054, with a percentage standard deviation of 2.8 per cent.

In old cultures (after 2 weeks) a debris of dead organisms accumulates in the tubes. To prevent the counting of dead ciliates, all of the liquid above the debris

in each tube was removed with a large pipette, and this was then sampled to determine the population of living organisms. The dead organisms that collect in the bottom of the tubes cytolyze very slowly and retain a recognizable form for a long time. This made possible the obtaining of a population figure which is an approximation of the total number of organisms that develop from the original inoculum. This figure, which we refer to as "total population," was obtained in the following way: As mentioned above, all the liquid above the debris in each tube was removed and sampled to determine the living population; after this sampling, the remainder of the culture with the living organisms was returned to the test tube containing the mass of dead organisms; the tube was shaken to mix the living and dead organisms; then the tube was immediately sampled, and all pieces recognizable as 50 per cent or more of a single organism were counted.

EFFECTS OF ADDED THIAMIN

Standard culture medium was prepared, inoculated, and divided into 10-cc. portions. These were divided into a control and an experimental series. Twenty gammas of thiamin hydrochloride per cubic centimeter of medium were added to each tube in the experimental series. Daily counts were made for the next several days, followed by counts at 3-day intervals. The results of the first experiment are shown graphically in Figure 1.

While the added thiamin did not change the fission rate during the logarithmic phase, it did increase the maximal population by maintaining the logarithmic phase for a longer period of time. The level of the experimental population was significantly above the control on the 3d, 4th, and 5th days, the difference in each case being greater than four times its standard error. The population level of the two series was about the same on the 6th day. The experimental series gave a higher count on both the 7th and 8th days, with a difference in each case of three times its standard error. The addition of this amount of thiamin resulted in a higher maximal population. It did not prevent the usual drop in population level which follows the maximum, but the initial drop was not to as low a level as that in the control series.

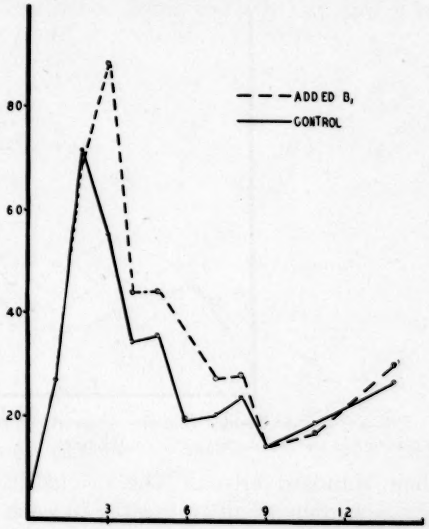

FIG. 1.—Effect of added thiamin on growth in the basic medium. Abscissa, time in days. Ordinate, organisms in thousands per cubic centimeter.

Experiment 2 was designed to test the effect of thiamin on conditioned medium. The basic medium was conditioned by growing *Tetrahymena* in it for 7 days. This medium was then cleared, reinoculated, and tubed by the standard procedure. The tubes were divided into a control series, A, and two experimental series, B and C. Thiamin chloride in the proportions of 10 γ per cubic centimeter were added to Series B and 20 γ per cubic centimeter to Series C. The tubes were sampled at intervals over a 21-day

period. The results for Series A and C are shown in Figure 2.

Here, also, the addition of thiamin produced a higher maximal population, and a higher level of population for several days following the maximum. The counts for both the B and C series, from the 3d through the 10th day, were significantly higher than the counts of the control tubes. The differences between the experimentals and controls, in this period, ranged from four to eleven times their standard errors. The counts in Series C were slightly greater in each case than those in Series B. The maximal density of the control series was not above the level at which such populations are maintained for a long period of time (cf. Expt. 5). While the addition of this amount of thiamin to conditioned medium did result in significant increases in the population, it did not make the conditioned medium equivalent to fresh medium.

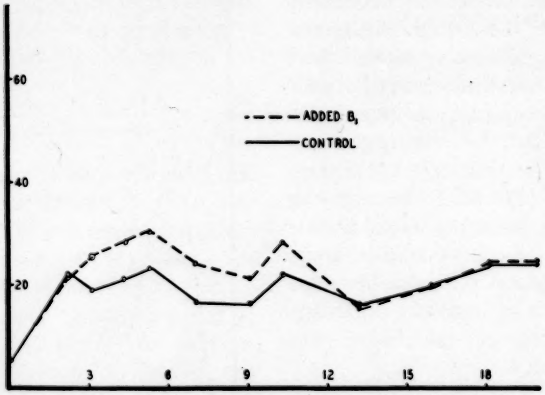

FIG. 2.—Effect of added thiamin on growth in conditioned medium. Abscissa, time in days. Ordinate, organisms in thousands per cubic centimeter.

EFFECTS OF ADDED THIAMIN AND RIBOFLAVIN

In Experiment 3 conditioned medium in which the ciliates had been growing for 15 days was used. Three series of tubes were started, as before. Nothing was added to Series A; 20 γ of thiamin per cubic centimeter of medium were added to Series B; and 20 γ of thiamin plus 20 γ of riboflavin per cubic centimeter of medium were added to Series C. The results, over a 20-day period, were very similar to those obtained in the second experiment. The use of medium which had been conditioned for a longer period did not result in appreciably lower population levels in the control series than those obtained in Experiment 2. The cultures containing thiamin alone had significantly higher populations from the 4th through the 11th day, the differences being greater than three times their standard errors. The counts in Series C, with both thiamin and riboflavin added, were significantly higher than the control counts from the 4th day on, with differences over four times their standard errors. The population level in the C series was significantly above that of the B series on the 13th day, with a difference of six times its standard error. This might be interpreted as an indication of a supplementary effect of riboflavin when added to conditioned medium with thiamin.

In the fourth experiment the basic medium was autoclaved for 2 hours. No test was made for the removal of vitamin B components, but such treatment should reduce the normal content. The pH of the medium was changed from the usual 6.8 to 6.2, indicating some hydrolysis of peptones. The medium was inoculated and tubed by the regular procedure. As in the preceding experiment, Series A was the control; to Series B were added 20 γ of thiamin per cubic centimeter of medium; and to Series C were added 20 γ of thiamin and 20 γ of riboflavin per cubic centimeter of medium. The data are given in Figure 3. Apparently the heat treatment did not deplete the medium very much. However, the last count in the control series, after 48 days, shows a very low population level. When this is compared with a count for about the same time interval in a similar series in the last experiment (cf. Table 1), it is much lower. The control series in the last experiment maintained a much higher level than this for a longer period of time. This may mean that heating did deplete the medium, as the medium in the last experiment was not so treated. The counts for both of the experimental series are significantly greater than the count for the control series at 2 days. The difference in each case is three times its standard error. There are practically no differences in the counts made on the 3d day. After that the B series gave population counts significantly greater than the controls on the 4th, 5th, 6th, 9th, 23d, 30th, and 48th days, with differences greater than three times their standard errors. The level in the C series was significantly above that in the control series from the 4th day on. The counts in the C series were significantly above those in the B series on the 12th, 16th, 30th, and 39th days, again indicating a possible supplementary effect of riboflavin when added to medium with thiamin.

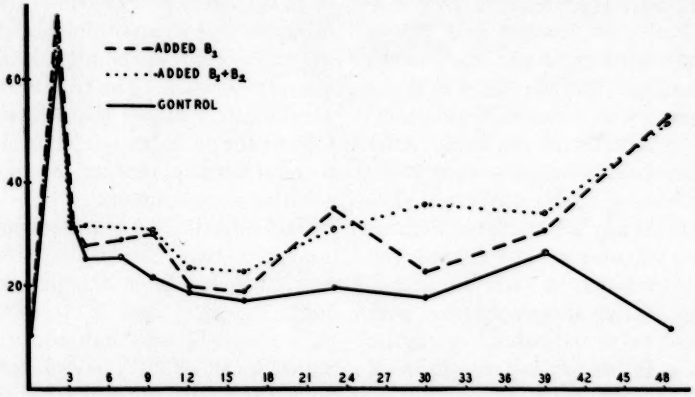

FIG. 3.—Effect of added thiamin and of thiamin plus riboflavin on growth in heat-treated medium. Abscissa, time in days. Ordinate, organisms in thousands per cubic centimeter.

NINETY-EIGHT-DAY EXPERIMENTS

The final samples of Series B and C in the preceding experiment indicated that these populations might be starting a second phase of increase. This and other observations suggested the need of following such populations over a much longer period of time. Experiment 5 was designed to do this and also to obtain information on the following questions:

(1) Will the addition of more thiamin produce greater differences? (2) Will certain other B components or combinations of them increase the population level more than thiamin and riboflavin?

In this experiment five series of twelve tubes each were set up by the standard procedure. Series A was the control; the tubes in Series B contained 100 γ of thiamin per cubic centimeter of medium; the tubes in Series C contained 20 γ of thiamin, 10 γ of riboflavin, and 5 γ of vitamin B_6 hydrochloride per cubic centimeter of medium; the tubes in Series D contained 20 γ of thiamin and 5 γ of para-aminobenzoic acid per cubic centimeter of medium; and the tubes in Series E contained 5γ of *p*-aminobenzoic acid per cubic centimeter of medium. After the first two counts samples were taken at approximately 10-day intervals. The results of this study are shown in Figures 4 and 5 and in Table 1. The figure given for living population in each nstance is the average of two sample counts, with the standard error indicated; the figure for total population in each case is based on a single sample count.

The heavy dosage of thiamin in Series B was not more effective in increasing the initial maximal phase than the dosage used in the first experiment. After the initial maximal phase the populations in this series showed no further effect of the thiamin until the high population increase after the 56th day. After this the B series gave much higher population counts than the controls, with differences over ten times their standard errors. It should be noted that the count for the B series on the 79th day was almost equal to the original high count on the 2d day.

The cultures in Series C, containing the thiamin-riboflavin-B_6 mixture, gave living population counts significantly above those of the control series, with the exception of the counts on the 56th day. There was some indication in the third and fourth experiments that riboflavin supplemented the effects of thiamin in maintaining a higher population level than that in control cultures, over long periods of time. It is not possible, without additional experiments, to determine the role of each vitamin in the results obtained in Series C. The counts in Series C were definitely above those in Series B from the 14th through the 46th day. This result might be due to the effects of riboflavin and pyridoxin together or to the effect of only one of them. Or it might be due to an inhibiting effect, during this period, of the high dosage of thiamin in Series B. The counts in Series B are definitely higher than those in Series C from the 79th day on. It is not possible to offer an explanation of this without additional experiments.

The addition of *p*-aminobenzoic acid produced two noticeable effects. The maximum levels of the populations in both Series D and E (2-day counts) were much higher than those found in Series A, B, or C. The differences were more than four times their standard errors. Also, the addition of *p*-aminobenzoic acid alone (Ser. E) produced what we refer to as an "exhausting effect" on the culture medium. The cultures in Series E had population counts significantly above the controls on the 35th and 56th days, with differences over eight times their standard errors; but the last two counts in this series were much below the controls. These cultures not only failed to show the secondary increase found in the other experimental series but they also died out more rapidly than the controls. The total population counts in this series, during the last part of the experiment, were much lower than those in any of the other series. The addition of thiamin with *p*-aminobenzoic acid (Ser. D) counteracted very largely this

exhausting effect. The cultures in Series D showed a higher secondary increase than that found in any other series. From the 64th day on, the counts in Series D the living population counts on the 79th and 86th days in Series D were almost equal to the original high count in this series.

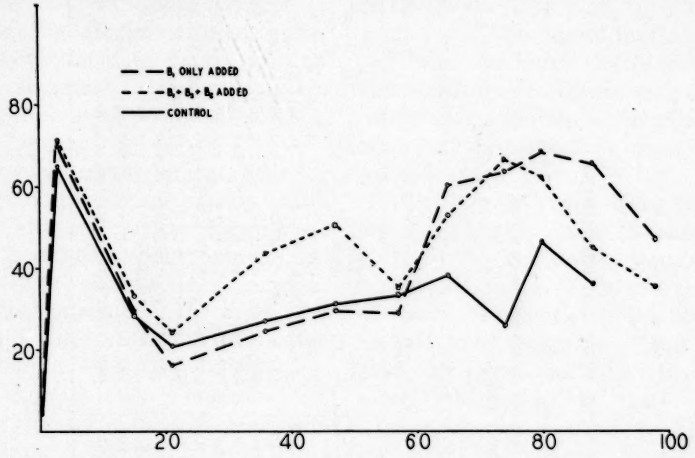

FIG. 4.—Population trends in Series A, B, and C. Abscissa, time in days. Ordinate, organisms in thousands per cubic centimeter.

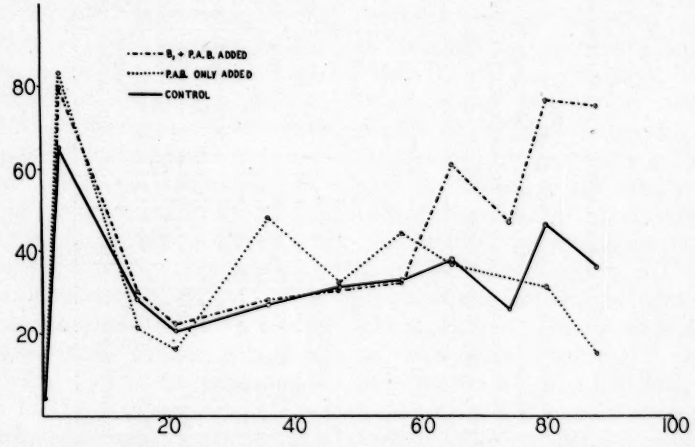

FIG. 5.—Population trends in Series A, D, and E. Abscissa, time in days. Ordinate, organisms in thousands per cubic centimeter.

were significantly above those in Series E, with differences greater than seventeen times their standard errors. The counts in Series D for this same period were also significantly above the controls. It should be noted here, also, that

An attempt was made to obtain further information on the effects of the various vitamins by using the "total population" counts already referred to. Total population counts (one count used instead of the average of two) paralleling

TABLE 1

TOTAL POPULATION VERSUS LIVING POPULATION FOR THE 98-DAY EXPERIMENT

Series A: Control
Series B: 100 γ per cubic centimeter of thiamin added.
Series C: 20 γ of thiamin, 10 γ of riboflavin, and 5 γ of vitamin B_6 hydrochloride per cubic centimeter added.
Series D: 20 γ of thiamin and 5 γ of para-aminobenzoic acid per cubic centimeter added.
Series E: 5 γ of para-aminobenzoic acid per cubic centimeter added.

TIME		SERIES A		SERIES B		SERIES C		SERIES D		SERIES E	
Days	Hours	Living	Total	Living	Total	Living	Total	Living	Total	Living	Total
0	0	4,230	4,230	4,230	4,230	4,230	4,230	4,230	4,230	4,230	4,230
2	14	65,178±130	65,178	69,821±741	69,821	71,250±1,638	71,250	79,821±1,356	79,821	83,036±913	83,036
14	22	28,482±569	352,678	29,286±995	402,678	34,107±467	339,928	30,268±60	372,321	21,607±1,037	363,393
21	2	20,714±747	370,536	16,161±775	437,500	24,375±804	476,785	22,500±112	479,464	16,250±682	411,607
35	19	27,232±763	498,214	24,732±173	758,928	43,661±1,047	655,357	28,214±1,438	848,214	48,036±480	492,857
46	20	31,357±1,316	773,214	29,715±1,188	953,571	50,554±101	798,214	30,822±215	798,214	32,679±1,699	800,000
56	19	33,214±1,627	926,786	29,357±733	1,189,286	33,143±1,690	975,000	32,679±1,274	976,786	44,393±44	562,500
64	19	38,464±77	1,046,429	60,429±846	807,143	52,821±2,007	910,714	61,071±427	737,500	37,071±889	666,071
73	17	26,035±1,119	1,103,571	63,857±702	1,346,429	66,536±332	591,071	47,036±987	1,182,143	Tube lost	
79	19	46,607±792	1,276,787	68,464±1,232	1,378,571	62,250±684	1,216,071	76,821±921	792,857	31,071±1,491	739,286
86	19	36,321±1,125	1,392,857	65,893±197	1,446,428	45,000±720	1,244,643	75,107±225	860,714	15,107±320	733,928
97	22	No samples left		47,054±940	1,517,860	35,625±997	1,646,429	22,321±624	1,275,000	No samples left	

those for the living populations were made throughout this experiment. The data on these counts are given in Table 1.

With the exception of Series E, there was a rather uniform rate of increases in total population in the different series. This was especially so in the control series. A comparison of the living and total population in the control series is shown in Figure 6.

These results seem to indicate that the rapid decline in living population which occurs at the end of the logarithmic phase of population growth is not so much the result of a decrease in fission rate as it is the result of the onset of a death rate much higher than the fission rate. As mentioned above, the cultures in Series E (with only *p*-aminobenzoic acid added) did not maintain the uniform rate of increase in total population found in the other series. After the 46th day there was a slow decrease in the total population in this series, whereas the total populations of the other series continued to increase. This relatively early cessation of increase in total population in Series E might be explained as due to a more rapid cytolysis of dead organisms were it not for the accompanying decreases in living population.

During the first 56 days of the experiment there was a uniform increase in total population in Series A, B, and C. The total population counts for this period in Series B were definitely higher than those in Series A and C, with the exception of the counts on the 35th day, when Series C had the highest count. From the 21st through the 56th days the counts in Series C were higher than the controls. Reference to the data in Table 1 shows that during this same period Series C had a significantly higher level of living population, while the living population in Series B was not above that of the control series between the 14th and 56th days. This would seem to indicate that thiamin, in this high concentration, maintains a more vigorous rate of fission, while the mixture of the three vitamins exerts its effect, in part, by slowing the death rate. The rate of increase in total population in Series D (thiamin and *p*-aminobenzoic acid added) seems to represent a combination of the effect of thiamin and the exhausting effect of *p*-aminobenzoic acid.

At certain intervals during the experiment noticeably large fluctuations in both living and total population occurred. Reference to Table 1 shows that in Series B the living population increased from 29,357 to 60,429 from the 56th day to the 64th day, while at the same time the total population dropped from 1,189,286 to 807,143; in Series C the living population increased from 33,143 to 66,536 between the 56th and 73d days, while the total population dropped from 976,786 to 737,500. Similar fluctuations occurred in Series D between the 73d and 79th days. These fluctuations can scarcely be assigned to chance deviations in sampling. They do not occur in all the series (e.g., there is no such fluctuation in the total population in the control series); and, furthermore, they occur in different series at the same time. Between the 56th and 64th days the increases in living population in Series B, C, and D were marked—almost to the original peaks; and during the same period the total population in each series showed a marked decrease. Such a second increase in living population as occurred in Series E took place much earlier, and there was no very marked secondary increase in the control series. These fluctuations, where decreases in total population are paralleled by increases in living population, are best understood when the data on living and total population in any one series are compared.

This point is illustrated for Series B in Figure 7. Here the living population is plotted twice: once on the same scale as the total population, so that the actual difference between living and total population may be seen, and once on a larger scale, so that the correlations in population fluctuations may be more clearly seen. A possible cause of these fluctuations will be discussed below.

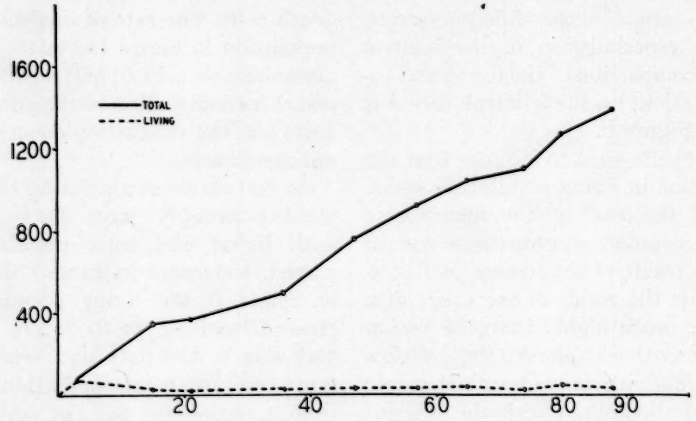

FIG. 6.—Comparison of total and living populations in the control series. Abscissa, time in days. Ordinate, organisms in thousands per cubic centimeter.

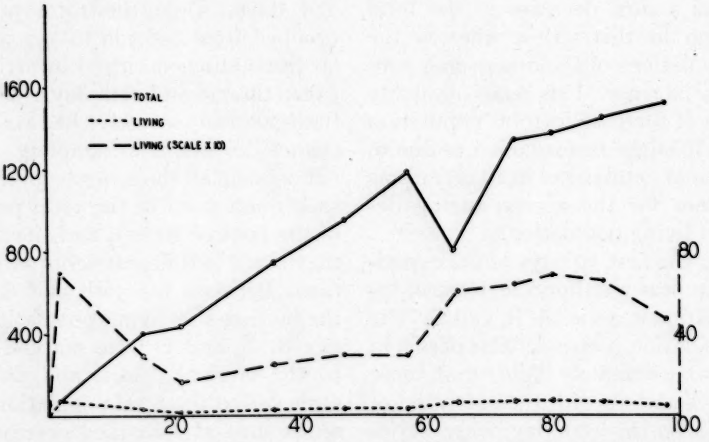

FIG. 7.—Comparison of total and living populations in Series B. "Living×10" shows the living population plotted on the right-hand ordinate. Abscissa, time in days. Ordinate, organisms in thousands per cubic centimeter.

DISCUSSION

A. and M. Lwoff (1937), working with *G. piriformis*, and Elliott (1939), working with *Colpidium striatum*, have demonstrated that thiamin must be present in the medium to obtain growth of these ciliates. Hall and Shottenfeld (1941) have shown that additions of thiamin to a casein-peptone medium will increase the period of logarithmic growth in *G.*

piriformis and thus increase the maximum level of the population and also decrease the death phase that follows the maximal phase. The results reported here on *T. geleii*, the same species as the two mentioned above or a very closely related species, grown in a proteose-peptone medium, substantiate the findings of Hall and Shottenfeld. The addition of 20 γ of thiamin per cubic centimeter of medium to conditioned medium produced an increase in the population level, but this amount of thiamin did not produce a maximum population as high as is normally obtained in fresh medium. This fact suggests that thiamin is only one of the factors controlling growth of these ciliates.

Hall (1942) has shown that riboflavin, as well as thiamin, is essential for growth of *C. campylum* in a de-ashed gelatin medium. Our results are not clear-cut on the effects of riboflavin and pyridoxin (B_6). The addition of riboflavin and thiamin in the third and fourth experiments gave results which suggest a supplementary effect of riboflavin, when added with thiamin, in maintaining a higher stationary level of living population. A similar result was obtained in the last experiment, where pyridoxin and riboflavin were added with thiamin. Further experiments are needed to determine the effects of riboflavin and pyridoxin on cultures of *T. geleii* growing in proteose-peptone medium over a long period.

The addition of *p*-aminobenzoic acid, as well as the addition of thiamin, caused an increase in the maximal level of the population. In fact, the addition of *p*-aminobenzoic acid alone produced a significantly higher maximal level than the addition of thiamin alone produced. However, there was no additive effect when these two vitamins were used together (Ser. D). There is no obvious reason for this, and more work is needed on this point. The effect which we have referred to as the "exhausting effect" on the medium by *p*-aminobenzoic acid, where the cultures with this vitamin, added alone, differed so from the other cultures following the initial maximal phase by dropping to the lowest population level (both living and total) of all the series, cannot be explained without additional experiments. To our knowledge, this is the first report of an effect of *p*-aminobenzoic acid on the growth of a protozoan population.

The term "total population" used here needs some comment. It is not used in the exact sense, because there was some cytolysis of dead organisms, as evidenced by fragments of organisms when counts were made and by the fluctuations in total population counts in Series B, C, and D; and some organisms were lost from each total population count in the taking of samples for living population counts. There is no way of knowing just how much cytolysis occurred in these cultures. The curve for total population of the control series, given in Figure 6, shows that there was practically a uniform rate of increase. When the living population, plotted on the same scale, is compared with the total population, the difference in numbers is quite striking. The living population, after the initial increase, remains quite stationary, while the total population increases at a fairly uniform rate. This indicates that the fission rate for the duration of the experiment was approximately the same and that the death rate during the long stationary phase of the living population was equal to the death rate. (The phase of accelerated death following the initial maximal phase is not shown in Fig. 6.) The experiments of Hall and Shottenfeld (1941) are comparable in many ways to the experiments reported here. They also followed trends in such populations for

long periods of time (about 75 days). Their data are much like the data given here for living populations. Either they failed to make clear in their paper that they separated living from dead organisms in their counts, or, in the medium which they used, cytolysis of dead organisms was quite rapid and there was no accumulation of dead organisms. It may well be that different results are obtained in different media in this regard. This finding suggests the need for distinguishing between living and dead organisms in future long-time experiments on protozoan populations.

The parallel fluctuations in total and living populations, where a decrease in total population is accompanied by an increase in living population, has been referred to. These fluctuations might be interpreted as being due to the cytolysis of large numbers of dead organisms at the time of decrease in total population and to the release of substances into the medium which causes an increase in the number of living organisms. The most striking of these parallel fluctuations occurred in the cultures to which thiamin had been added.

The general trend of the control series in the 98-day experiment, with a high maximal phase, a phase of accelerated death, and a long stationary phase, followed by a drop to a very low level, is quite similar to the trend reported by Hall and Shottenfeld. The marked secondary increases in living population in Series B, C, and D were not present in any of the experiments of Hall and Shottenfeld and should be attributed to the added thiamin. To make comparison easy, a purely synthetic medium of known constituents is clearly needed for such studies. The vitamin content of the medium is important in the study of growth of such populations, and we know but little about the effects of the various vitamins. A method is now available for vitamin assay of such culture media (e.g., Beadle and Tatum, 1941). Until it is possible to culture successfully such organisms on a purely synthetic medium, much might be learned about the effects of various vitamins by comparing results with different media which have been assayed for their content of the various vitamins.

These experiments have some bearing on the problem of the cessation of growth in cultures in relation to accumulated waste products. If by "cessation of growth" one means the downward trend of the living population which follows the maximal peak, the data presented here seem to indicate that this is due more to a high death rate than to a greatly reduced fission rate. These data indicate that added thiamin increases the maximal population by lengthening the logarithmic phase of growth. The results with *p*-aminobenzoic acid added suggest that the higher maximal population produced is due to an increase in the fission rate. However, more experiments are needed on this point. The cultures in Series C of the last experiment, containing thiamin, riboflavin, and pyridoxin, had higher levels of living populations than the controls. Can this result be due to a detoxifying effect of the vitamins? Also, can the higher maximal populations produced by thiamin alone be due to a detoxifying effect? The secondary increases in population, almost to the original peaks, found in Series B, C, and D of the last experiment, indicate that great increases in living population may occur after 64 days. In these three cases thiamin had been added to the medium, and in all of them a considerable drop in total population occurred concurrently with the increases in living population. At least three conditions seem to be associated here: added thiamin, decreases

in total populations at about the same time in different series resulting from the cytolysis of dead organisms, and marked increases in the living populations. The exact causal relationship cannot be stated. Obviously, more work is needed to clear up this question and those raised above.

SUMMARY

1. The addition of thiamin to fresh proteose-peptone medium produced a higher maximum population. However, the addition of thiamin did not cause an increase in the fission rate during the logarithmic phase of growth.

2. The addition of thiamin to conditioned proteose-peptone medium produced a higher maximum population. This maximum, however, was not equal to that obtained in fresh, untreated medium.

3. All of the long-time cultures containing added thiamin had secondary increases in population, almost to the original peaks, after 64 days.

4. Cultures with a mixture of thiamin, riboflavin, and pyridoxin added maintained the highest level of living population.

5. Cultures with para-aminobenzoic acid added had higher maximum populations than those obtained under other conditions. This may be due to an increase in the fission rate. Cultures with only *p*-aminobenzoic acid added died out sooner than the other types of cultures studied. The addition of thiamin with *p*-aminobenzoic acid prevented this early dying-out.

6. A method for comparing living and total population is described. In all of the cultures except those with only *p*-aminobenzoic acid added there was a fairly uniform increase in total population for over 90 days.

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THE PRODUCTION OF GROWTH SUBSTANCE IN COLPIDIUM STRIATUM (STOKES)[1]

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THE conclusion that *Enchelys farcimen* secretes a substance which accelerates reproduction was made by Robertson (1921*a*, *b*, 1922, 1923, and 1924). This conclusion is supported by Yocum (1928) in reference to *Oxytricha*, Petersen (1929) and Dimitrowa (1932) in reference to *Paramecium caudatum*, Mast and Pace (1938) in reference to *Chilomonas paramecium*, and Kidder (1941) in reference to *Tetrahymena geleii*. But it is not supported by Cutler and Crump (1923*a*, *b*, 1924, and 1925) in reference to *Colpidium colpoda;* Woodruff (1911 and 1913) and Greenleaf (1926) in reference to *Paramecium aurelia*, *Paramecium caudatum*, *Pleurotricha lanceolata*, and *Stylonychia pustulata;* Myers (1927) in reference to *Paramecium caudatum;* Johnson (1933) in reference to *Oxytricha fallax;* Phelps (1935 and 1936) in reference to *Glaucoma pyriformis;* and Johnson and Hardin (1938) in reference to *Paramecium multimicronucleatum*.

Johnson and Phelps maintain that the contradictory results obtained by previous investigators are largely, if not entirely, due to differences in the amount and kind of food available rather than to the effect of substances produced by the organisms studied. It is consequently evident that the conclusions concerning the production of growth substance in Protozoa are equivocal and that more experiments under thoroughly controlled environmental conditions are highly desirable.

MATERIALS AND METHODS

A sterile clone culture of *Colpidium striatum*[2] was established from a wild specimen by means of the Parpart (1928) method as modified by Kidder, Lilly, and Claff (1940) and grown in sterile 3 per cent Difco proteose-peptone (Elliott, 1933) in triple-distilled water (Mast and Pace, 1933) in 125-cc. flasks. The culture fluid was sterilized by autoclaving for 20 minutes at 20 pounds pressure. It was used throughout, except in one experiment described in detail below. All glassware used was Pyrex glass. It was cleaned and sterilized for experimental use according to the method used by Mast and Pace (1933).

Since proteose-peptone fluid will support growth of bacteria, molds, and other microörganisms as well as *Colpidia*, special precautions had to be taken at all times during the experiments to prevent contamination. All manipulations were consequently performed in a boxlike hood constructed of wood. The hood (95 cm. long, 70 cm. deep, and 75 cm.

[1] The writer takes pleasure in acknowledging his great indebtedness to Professor S. O. Mast for kind encouragement and criticism during the course of the investigation and the preparation of the manuscript.

[2] The organisms investigated correspond with the description of those designated *Colpidium striatum* by Stokes. Furgason (1940) united these with a number of similar forms under the name *Tetrahymena geleii*. He presents strong support in favor of the identity of the forms united, but I think the nomenclature proposed should be extensively discussed before it is accepted.

high) was closed and sealed on all sides except one. The open side, through which the operator worked, was closed when not in use by a double layer of rubberized photographer's cloth. The inner walls of the hood were painted with copper sulphate, and the bottom of it was covered with linoleum and washed biweekly with 80 per cent alcohol. All observations were made with a Bausch and Lomb binocular dissecting microscope, which was kept in the hood at all times. Difco nutrient agar plates exposed from time to time in the hood during manipulations rarely showed bacterial growth, only occasionally a mold colony formed, and only a very small percentage of the cultures used in the experiments became contaminated.

From the sterile clone culture in the flasks, subcultures were started in test tubes, each containing 20 cc. of fluid. These were subcultured from time to time so that vigorous sterile cultures were continuously on hand for nearly 9 months. The *Colpidia* used were obtained from slide cultures made by transferring 1 *Colpidium* from a test-tube culture to 0.1 cc. of culture fluid in a depression on a slide in a Petri dish and leaving it 24 hours or more. All experimental cultures were kept in a water bath at 24.5° ± 0.4° C. They were examined from time to time for turbidity, and samples were plated out on nutrient agar and incubated for 10 days. Cultures which became contaminated were discarded.

Individual *Colpidia* were transferred by means of Pyrex-glass capillary pipettes, which had been sterilized in test tubes in an oven. The bulbs were removed from the pipettes and the opening plugged with cotton before they were sterilized. Several experimental procedures were used; these are described in detail below.

RELATION BETWEEN THE NUMBER OF *Colpidia* IN A GIVEN VOLUME OF CULTURE FLUID AND RATE OF REPRODUCTION

Numerous *Colpidia* from four 24-hour slide cultures were transferred to each of 8 depressions, each containing 0.1 cc. sterile proteose-peptone culture fluid, and left 24 hours. The *Colpidia* produced were then transferred successively through four 0.1-cc. portions of fresh sterile proteose-peptone culture fluid so as to remove the old fluid. Then 0.1 cc. of the same kind of culture fluid[3] was put into each of 16 depressions on slides in Petri dishes, each of which contained 5 cc. sterile tap water to prevent evaporation, after which 1 of the *Colpidia* was put into each of 4 of the depressions, 5 into each of 4, 25 into each of 4, and 50 into each of 4. The Petri dishes were now put into a water bath at 24.5° ± 0.4° C. and left 12 hours. Then 1 *Colpidium* was removed from each of the depressions in Group 1, 5 from each in Group 2, 25 from each in Group 3, and 50 from each in Group 4, passed through four 0.1-cc. portions of fresh culture fluid so as to remove the old fluid, and put respectively into 0.1 cc. fresh sterile culture fluid. The remaining *Colpidia* were killed with acetic acid vapor and the number in each depression recorded. This process was repeated twelve times. The results obtained are presented in Table 1.

This table shows that the average number of *Colpidia* produced from each one introduced in 12 hours was 7.7 with

[3] The stock proteose-peptone culture fluid was kept in 125-cc. Pyrex-glass flasks. This was frequently pasteurized by heating it to 85° C. and renewed from time to time, owing to slight evaporation during pasteurization. The hydrogen-ion concentration was kept at pH 6.2.

1 in 0.1 cc. culture fluid, 10.5 with 5 in 0.1 cc., 7.2 with 25 in 0.1 cc., and 6 with 50 in 0.1 cc. The results obtained show, therefore, that, as the number of *Colpidia* per given volume of culture fluid increases, the rate of reproduction increases to a maximum and then decreases and that the optimum conditions for growth lies in the vicinity of 5 *Colpidia* per 0.1 cc. culture fluid.

TABLE 1

RELATION BETWEEN THE NUMBER OF *Colpidia* IN A GIVEN VOLUME OF CULTURE FLUID (0.1 CC.) AND RATE OF REPRODUCTION

One *Colpidium* was put into each depression in Group 1; 5 into each of Group 2; 25 into each of Group 3; 50 into each of Group 4. Temperature 24.5° ± 0.4° C.; hydrogen-ion concentration, pH 6.2.

Designation of Group and Number of *Colpidia* Introduced	The Average Number of *Colpidia* Produced in 12 Hours from Each *Colpidium* Introduced — Designation of Tests													Total Average	Standard Error
	1	2	3	4	5	6	7	8	9	10	11	12	13		
1 (1)...	10	6	7	9	8	9	8	7	8	7	11	7	6	7.7	±.199
	7	7	7	8	12	8	8	8	8	8	6	9	7		
	8	8	8	7	5	8	8	8	7	6	7	8	8		
	11	4	8	8	8	6	8	8	7	.....	8	7	7		
2 (5)...	9.8	5.6	8.2	13.6	12.0	11.0	8.6	7.6	9.4	14.2	14.6	16.2	10.6	10.5	±.335
	11.2	6.2	10.6	13.8	8.4	9.4	10.2	11.2	7.6	8.2	8.2	12.8	13.4		
	10.6	7.4	9.2	14.4	11.4	8.4	10.4	7.0	8.0	11.2	12.2	10.2	9.6		
	12.8	8.2	6.6	13.6	11.0	9.6	13.4	10.4	10.0	11.8	13.0	11.8	12.6		
3 (25)..	9.4	5.5	8.9	9.2	5.6	5.2	5.7	5.1	7.8	7.1	8.1	7.2	6.1	7.2	±.232
	9.1	6.1	7.8	8.7	6.1	6.0	6.2	5.7	6.8	7.7	7.8	5.9	6.9		
	11.4	5.2	7.5	8.4	9.3	.....	6.4	5.6	6.4	5.4	8.4	7.9	5.9		
	10.4	5.3	8.3	9.5	6.3	.....	.....	6.0	5.8	9.9	6.5	9.2	6.9		
4 (50)..	7.9	7.6	3.5	7.8	5.6	4.9	3.9	2.6	6.1	5.6	6.4	5.1	6.0	6.0	±.263
	10.5	5.5	5.4	6.4	4.7	9.1	5.2	5.8	5.7	3.2	6.5	6.2	3.7		
	10.6	6.0	5.0	.....	6.8	5.9	5.7	5.4	7.9	4.8	5.3	7.4	5.9		
	8.8	4.8	7.4	.....	5.2	11.2	4.8	6.8	4.9	3.4	5.6	6.4	6.1		

RELATION BETWEEN THE VOLUME OF CULTURE FLUID CONTAINING A GIVEN NUMBER OF *Colpidia* AND RATE OF REPRODUCTION

The methods used in this experiment were the same as those used in the preceding, except that a given number of *Colpidia* were introduced into different volumes of culture fluid in place of the reverse, and the process was repeated nine, instead of twelve, times, and in each test the *Colpidia* used were taken from different depressions than in the preceding test, as follows: in the second test from those in Group 10, in the fifth from those in Group 3, in the sixth from those in Group 5, in the eighth from those in Group 9, in the tenth from those in Group 6, and in the third, fourth, seventh, and ninth from those in all the groups, one from each depression. The results obtained are presented in Table 2.

This table shows that, as the volume decreased, the average number of *Colpidia* produced from each one introduced

TABLE 2

RELATION BETWEEN THE VOLUME OF CULTURE FLUID CONTAINING A GIVEN NUMBER OF *Colpidia* AND THE RATE OF REPRODUCTION

One *Colpidium* put into each depression. Temperature 24.5° ± 0.4° C.; hydrogen-ion concentration, pH 6.2.

Designation of Groups	Volume of Fluid in Cc. in Each Depression	Total Number of *Colpidia* Produced from 1 in Each Depression in 24 Hours — Number of Tests										Total Average	Standard Error
		1	2	3	4	5	6	7	8	9	10		
1.....	0.4	58	21	21	25	8	60	26	9	29	52	30.0	±2.17
		34	31	25	.....	9	.....	27	8	30	41		
		39	35	57	22	20	51	23	24	39	26		
		23	30	34	33	.....	38	28	21	29	23		
2.....	0.3	63	26	32	.....	16	73	29	55	.....	48	35.0	±2.70
		33	28	56	27	25	84	60	40	31	...		
		31	29	32	29	12	49	45	32	35	12		
		21	16	29	20	18	23	30	29	30	46		
3.....	0.2	54	36	27	16	22	65	46	46	22	18	40.7	±2.40
		56	17	60	32	.....	44	53	55	27	44		
		30	19	31	32	17	64	37	46	34	47		
		48	39	53	39	.....	59	55	42	45	69		
4.....	0.1	38	14	30	25	39	50	47	60	28	47	47.0	±2.80
		60	26	57	22	29	55	53	44	34	62		
		31	36	33	28	53	61	48	63	62	95		
		44	51	59	30	60	92	58	.....	60	53		
5.....	0.075	76	64	.....	29	43	62	74	64	31	53	49.6	±2.90
		36	32	32	56	31	97	53	56	58	56		
		62	33	55	30	37	46	36	37	29	32		
		73	60	65	38	28	78	77	.....	34	30		
6.....	0.05	32	33	61	51	.....	84	56	36	39	45	53.8	±2.70
		57	36	43	28	41	54	64	61	33	58		
		59	41	88	22	55	89	63	72	66	63		
		67	66	76	31	36	56	55	59	58	64		
7.....	0.0375	47	59	71	22	33	55	59	56	31	65	48.9	±2.25
		66	31	.....	32	54	63	57	41	33	53		
		60	57	62	27	47	69	31	57	35	43		
		71	60	56	30	60	61	51	36	29	36		
8.....	0.025	53	54	54	.....	56	55	58	47	28	44	48.5	±1.80
		60	33	.....	.....	57	58	61	32	31	41		
		44	60	59	61	59	58	28	38	54	48		
		35	56	67	37	53	52	43	48	35	36		
9.....	0.0125	59	45	34	19	56	34	.....	54	29	15	42.4	±2.36
		62	69	60	31	54	59	34	48	15	34		
		32	42	63	33	58	49	.....	45	26	28		
		56	35	58	28	52	52	31	46	33	32		
10.....	0.006	47	37	42	27	29	33	28	52	39	31	37.8	±2.08
		26	17	59	29	28	30	70	61	27	38		
		41	38	56	20	49	57	33	30	33	25		
		29	29	69	27	31	48	36	41	24	46		
11.....	0.003	27	48	38	39	22	33	46	50	38	12	30.1	±1.03
		30	17	46	24	27	.....	33	41	30	11		
		31	46	22	19	29	.....	38	20	27	24		
		25	26	26	41	28	29	.....	22	.....	17		

increased from 30.0 specimens in 0.4 cc. to a maximum of 53.8 in 0.05 cc. and then decreased to 30.1 in 0.003 cc. It shows, consequently, that the rate of reproduction with 1 *Colpidium* in 0.05 cc. culture fluid was 79.5 per cent higher than it was with 1 *Colpidium* in 0.4 cc. and that, irrespective of the source from which the *Colpidia* were taken, the rate of reproduction increased to a maximum as the volume decreased and then it decreased.

Table 2 shows that there was marked variation in the number of *Colpidia* produced in different days in a given volume of culture fluid. It shows, for example, that in 0.4 cc. the average number of specimens produced per day in the 4 depressions in a test varied from 12.3 to 49.7. One of the factors which undoubtedly caused this variation and consequently the high standard errors is that the age of the specimens put into the depressions varied, for it was impossible to select specimens of the same age. It was found by standard methods that, owing to the high standard errors, there is no significant difference between the averages for adjoining groups but that there is a significant difference between the averages for groups somewhat separated, i.e., 1 and 4, 2 and 5, 3 and 6, 11 and 8, 10 and 7, and 9 and 6. Moreover, the increases in the magnitude of the averages to a maximum are perfectly consistent from group to group, as are also the decreases from the maximum to a minimum.

The results obtained, therefore, strongly support the conclusion reached in the preceding experiment, namely, that, as the volume of culture fluid per *Colpidium* decreases, the rate of reproduction increases to a maximum and then decreases. This conclusion is in full harmony with that reached by Mast and Pace (1938) in observations on *Chilomonas paramecium*. The results also show that the optimum volume per individual is approximately 0.05 cc. But they do not show what factors are involved in the correlation presented above. The following experiments concern this.

THE EFFECT ON THE RATE OF REPRODUCTION OF CHANGES IN THE CULTURE FLUID PRODUCED BY THE METABOLISM OF *Colpidia*

Numerous *Colpidia* were taken at random from 24-hour slide cultures and transferred successively through four 0.1-cc. portions of sterile culture fluid. Then 0.2 cc. of sterile culture fluid was put into each of 48 depressions on slides in Petri dishes, and 1 of the *Colpidia* was put into each of 12 of the depressions, 5 into each of 12, 10 into each of 12, and 25 into each of 12. The Petri dishes were then put into a water bath at 24.5° ± 0.4° C. and left 4 hours, after which the temperature was reduced nearly to 0° and held 15 minutes in order to inactivate the *Colpidia*. Then as much of the culture fluid as possible (at least 0.1 cc.) was transferred, with a capillary pipette, from each of the 12 depressions (with 1 *Colpidium*) to a sterile Columbia dish in a Petri dish. Then 0.1 cc. of this fluid was put into each of 12 depressions in Petri dishes, 0.1 cc. fresh culture fluid into each of 12 depressions, and 1 *Colpidium*, which had just divided, added to each of the 24 depressions. All the Petri dishes were put into the water bath, all the specimens in them examined at short intervals, and the time required for the first division in each recorded. This (excepting the fresh culture fluid) was repeated with the other three groups of 12 depressions, which had received, respectively 5, 10, and 25 *Colpidia* per 0.2 cc. The entire process was then repeated twice exactly as described, and twice using 100 and

500, respectively, in 0.2 cc. culture fluid instead of 1, 5, 10, and 25.

The young *Colpidia* used in this experiment were obtained as follows: Forty-eight hours before they were needed, 0.05 cc. fresh sterile culture fluid was put into each of 2 depressions in Petri dishes and 2 *Colpidia* from a 24-hour-slide culture added to each. The dishes were then put into the water bath and left 24 hours. Then 5 of the *Colpidia* produced were transferred to each of 12 depressions containing 0.1 cc. culture fluid each. These were put into the water bath and left 24 hours, after which they were, at short intervals, examined for specimens in the process of fission. As soon as one was found, it was passed through four 0.05-cc. portions of fresh culture fluid so as to eliminate the old culture fluid and observed continuously until fission was complete. The *Colpidia* used were therefore very nearly the same in age and physiological condition, and the measurements were consequently more nearly accurate than in the preceding experiment, in which the *Colpidia* used varied considerably. The results obtained are presented in Table 3.[4]

Table 3 shows that the average time required for the first division in fresh culture fluid was 6.42 hours and that, as the extent of metabolism in this fluid increased, the time decreased to 5.11 hours in fluid in which 5 *Colpidia* per 0.2 cc. had been for 4 hours and then increased to 8.46 hours in fluid in which 500 *Colpidia* per 0.2 cc. had been for 4 hours. The difference between the average for fresh culture fluid and the minimum average is statistically significant, as is also

TABLE 3

THE EFFECT ON THE RATE OF REPRODUCTION OF CHANGES IN CULTURE FLUID PRODUCED BY METABOLISM IN *Colpidium striatum*

(Temperature 24.5° ±0.4° C.)

Culture Fluid	No. of *Colpidia* (Each in 0.1 Cc. Culture Fluid)	Time in Hours Required for First Division					Total Average	Standard Error
		Average						
		Test A	Test B	Test C	Test D	Test E		
Proteose-peptone	63	6.33	6.14	6.68	6.74	6.25	6.42	±.0679
Same, in which 1 *Colpidium* per 0.2 cc. had been for 4 hours	36	6.16	5.80	5.83	.....	.....	5.93	±.063
Same, except 5 *Colpidia* per 0.2 cc.	36	4.80	5.46	5.05	.....	.....	5.11	±.056
Same, except 10 *Colpidia* per 0.2 cc.	36	5.88	5.89	5.42	.....	.....	5.92	±.0498
Same, except 25 *Colpidia* per 0.2 cc.	36	5.81	6.08	6.33	.....	.....	6.07	±.104
Same, except 100 *Colpidia* per 0.2 cc.	53	.....	.....	.....	7.14	7.23	7.16	±.0714
Same, except 500 *Colpidia* per 0.2 cc.	49	.....	.....	.....	9.21	7.38	8.46	±.148

[4] The author wishes to point out the fact that in the two preceding experiments the number of *Colpidia* produced from each 1 introduced were counted after 12 and 24 hours, respectively. These cultures were frequently examined after they were started, and it was observed that more time was required for the first and second divisions than for succeeding divisions and that the optimum conditions for growth in these cultures did not obtain until after the cultures were several hours old. At the present time the author does not have sufficient results to present in order to show when the accelerating effect takes place in a 12- or 24-hour culture. I hope to investigate this problem further and report on it in a subsequent paper.

the difference between the minimum average and the maximum average.

Consequently, the results presented show that *Colpidia* produce changes in their culture fluid which profoundly affect their rate of reproduction, that moderate changes cause marked increases and extensive changes marked decreases in rate of reproduction, and that these changes in the rate of reproduction cannot be due to the immediate effect of the *Colpidia* on one another.

The changes in the culture fluid may obviously be due to the production of substance by the *Colpidia* or consumption of substance or both. The increase in rate of reproduction must be due to the production of growth-promoting substances, for the compounds in the culture fluid are in optimum concentration; but it is evident that the decrease may be due either to production of a growth-retarding substance or to reduction in the concentration of some of the compounds in the culture fluid used as food. If it is the latter, the rate of reproduction should be closely correlated with the concentration of the culture fluid. The following experiment concerns this.

RELATION BETWEEN CONCENTRATION OF CULTURE FLUID AND RATE OF REPRODUCTION

The methods used were the same as those used in the preceding experiment. The results obtained are presented in Table 4. These results show that no divisions were obtained in distilled water and that, as the concentration of the culture fluid in distilled water increased, the average time required for the first division decreased from 8.9 hours at 0.006 per cent to 6.21 hours at 0.03 per cent, then remained nearly constant until 4 per cent was reached, then increased to 8.48 hours at 6 per cent—the highest concentration tested. These results consequently show that the time required for the first division is practically unchanged, even if the available food supply is reduced from 4 per cent to 0.03 per cent, or almost 100 times.

In the preceding experiment it was found that after 500 *Colpidia* had been in 0.2 cc. of 2 per cent proteose-peptone solution for 4 hours the average time required for division increased from 6.42 to 8.46 hours, i.e., nearly 2 hours. Table 4 shows that, if this decrease in the rate of reproduction was due to the consumption of proteose-peptone, the 500 *Colpidia* must have consumed in 4 hours enough to reduce the concentration from 2 to nearly 0.006 per cent. This is obviously impossible. The decrease in the rate of reproduction must, therefore, have been due to the production of a growth-retarding substance. This conclusion is in

TABLE 4

THE RELATION BETWEEN THE CONCENTRATION OF FOOD (PROTEOSE-PEPTONE) IN THE CULTURE FLUID AND THE RATE OF REPRODUCTION OF *Colpidium striatum*

(Temperature 24.5° ± 0.4° C.)

Grams Proteose-Peptone per 100 Cc. Water	Total No. of *Colpidia* (Each in 0.05 Cc. Culture Fluid)	Time in Hours Required for the First Division	Standard Error
		Total Average	
0.000.....	32	Dead after 4 hours, no divisions	.....
0.006.....	32	8.90	±.279
0.03.....	32	6.21	±.133
0.1.....	32	6.06	±.105
0.5.....	32	5.88	±.0751
1.....	32	6.37	±.121
2.....	32	6.01	±.107
4.....	32	7.31	±.184
6.....	32	8.48	±.118

accord with that reached by Mast and Pace (1938) in observations on *Chilomonas paramecium* and by Kidder (1941) in observations on *Tetrahymena geleii*. Mast and Pace, however, contend that the reduction in rate of reproduction is due to high concentration of the substance which in low concentration causes increase in rate of reproduction, in other words, that only one kind of substance is produced and that different effects are correlated with its concentration. Kidder, however, maintains that, if *Tetrahymena* is removed from 60-hour-old cultures by means of the centrifuge, the fluid accelerates the rate of reproduction, but if it is removed by means of a filter it retards the rate of reproduction. He concludes that two different substances are produced, one which promotes growth and another which retards it.

If the retardation in rate of reproduction in old culture fluid is due to high concentration of the substance which in low concentration accelerates it, dilution of old with fresh culture fluid should cause an increase in rate of reproduction. But, if the old culture fluid contains a growth-promoting and a growth-regarding substance, there should be no increase in the rate of reproduction. The following experiment concerns these propositions.

THE EFFECT OF DILUTING OLD WITH FRESH CULTURE FLUID ON THE RATE OF REPRODUCTION OF *Colpidium striatum*

The methods used were the same as those used in the preceding experiments. The results obtained are presented in Table 5, which shows that, as the concentration of old culture fluid increased, the average time required for the first division decreased from 5.35 hours in fresh culture fluid to a minimum of 5.01 hours in 22 cc. old/10 cc. fresh fluid and then increased to 8.18 hours in old culture fluid. That is, it shows that, as old culture fluid is diluted, the rate of reproduction increases to a maximum and then decreases. These results therefore show that but one substance is active in producing acceleration and retardation in rate of reproduction in *Colpidia*—accelerating it in low concentration and retarding it in high concentration. The next experiments concern the nature of the substance produced by *Colpidia*.

TABLE 5

THE RELATION BETWEEN THE CONCENTRATION OF 8-DAY-OLD CULTURE FLUID IN FRESH CULTURE FLUID AND THE RATE OF REPRODUCTION OF *Colpidium striatum*

(Temperature 24.5° ± 0.4° C.)

Ratio of Fresh to Old Culture Fluid	Total No. *Colpidia* (Each in 0.05 Cc. Fluid)	Time in Hours Required for First Division		Total Average	Standard Error
		Average			
		Test A	Test B		
0/10.....	40	4.94	5.75	5.35	±.110
2/10.....	40	4.58	5.44	5.01	±.100
4/10.....	40	5.55	6.47	6.01	±.115
6/10.....	40	6.11	6.70	6.40	±.110
8/10.....	40	6.69	7.31	7.00	±.132
10/10.....	40	7.97	8.39	8.18	±.128

THE EFFECT OF HEAT ON THE CHANGES PRODUCED IN CULTURE FLUID BY *Colpidia*

Mast and Pace (1938) in observations on *Chilomonas paramecium* found that, if old culture fluid which inhibits reproduction is subjected to 100° C. for different periods of time, the rate of reproduction in it increases with time from zero to a maximum and then decreases to that which obtains in fresh culture fluid and that this requires about 90 minutes. Kidder (1941) maintains that, if culture fluid in which *Tetrahymena geleii* has grown until growth is retarded is subject-

ed to 100° C., it is restored to normal and that this requires only 5 minutes. The following experiments were performed to ascertain the effect of heat on the growth substance produced by *Colpidium striatum*.

EXPERIMENT I. CULTURE FLUID 3 MONTHS OLD

Culture fluid in which *Colpidia* had grown for 3 months was centrifuged, and 1.5 cc. of the fluid without *Colpidia* was put into each of 2 sterile pyrex-glass tubes, and the same amount of fresh fluid was put into each of 2 other tubes. Then 1 of the former and 1 of the latter were suspended in a hot water bath maintained at 100° C. for 2 hours. The rate of reproduction of *Colpidia* in solution from each of the 4 tubes was then measured as described above and the entire process repeated. The results obtained are presented in Table 6, Experiment I.

These results show that for the first division an average of 5.95 hours was required in fresh culture fluid and very nearly the same time (6.05 hours) in this fluid after it had been subjected to 100° C. for 2 hours but that an average of 16.86 hours was required in this fluid after *Colpidia* had grown in it 3 months and 15.85 hours after this fluid had been subjected to 100° C. for 2 hours. It consequently shows that the time required for the first division is very much longer in old (3-month) than in fresh culture fluid and that heating causes practically no change in fresh, but a reduction of a little more than 1 hour in old culture fluid. Mathematical treatment of the averages indicates, however, that this decrease is not statistically significant.

EXPERIMENT II. CULTURE FLUID 2, 4, AND 8 DAYS OLD

Numerous *Colpidia* were taken from 16 slide cultures and transferred successively through four 0.1-cc. portions of fresh culture fluid so as to remove the old culture fluid. Then 100 of these were put into each of 3 Columbia dishes (in Petri dishes) containing 1 cc. fresh culture fluid and left at 24.5 ± 0.4° C. in the water bath for 8 days. Four and 6 days later this was repeated, but the *Colpidia* were left in the water bath only 4 and 2 days, respectively, in place of 8. There were then at hand 3 sterile cultures in Columbia dishes, one 2 days old, one 4 days old, and one 8 days old.[5] The *Colpidia* were now removed from each of these cultures by means of the centrifuge; and half of the fluid in each and an equal portion of fresh culture fluid were subjected to heat as described above, except that the temperature was maintained at 100° C. for 3 hours instead of 2. The rate of reproduction of *Colpidia* in each of these 7 different portions of culture fluid, and also in fresh culture fluid, was then measured as described above. The entire process was then repeated twice. The results obtained are presented in Table 6, Experiment II.

These results show that for the first division an average of 5.65 hours was required with *Colpidia* in fresh culture fluid and nearly the same (5.85 hours) in fresh culture fluid which had been heated, but that in 2-, 4-, and 8-day-old culture fluid it required 6.46, 8.08, and 9.96 hours, respectively, and that heat[6] caused considerable decrease in the time required in these solutions, i.e., 24, 52, and 109 minutes, respectively. Mathematical treatment of the results shows that the differ-

[5] The optimum condition for growth was attained in these cultures after they were 24 hours old. Consequently, after they became older than 24 hours, the solution in them retarded growth.

[6] The 8-day, 4-day, and 2-day fluid became slightly milky after heating for 3 hours, but this did not take place in fresh culture fluid. This milky appearance was just perceptible. It will be dealt with later.

ences between the time required for the first division in successive pairs of the four solutions are all statistically significant and that the reduction in the time required in the old solutions (caused by heating) is also statistically significant. The results consequently show that subjecting old culture fluid to 100° C. for 3 hours reduces its retarding effect on the rate of reproduction but not to zero.

TABLE 6

THE EFFECT OF HEAT ON CULTURE FLUID IN WHICH *Colpidia* HAD GROWN FOR DIFFERENT LENGTHS OF TIME, AS INDICATED BY THE RATE OF REPRODUCTION OF *Colpidia* IN IT

(Temperature 24.5° ± 0.4° C.)

Proteose-Peptone Culture Fluid	No. of *Colpidia* (Each in 0.05 Cc. Fluid)	Time in Hours Required for First Division			Total Average	Standard Error
		Average				
		Test A	Test B	Test C		
		Experiment I				
Fresh	40	5.81	6.09	.....	5.95	± .1156
Same after 2 hours at 100° C.	32	5.97	6.31	.....	6.05	± .1045
3 months old	26	13.99	20.39	.....	16.86	± .643
Same after 2 hours at 100° C.	30	13.36	18.69	.....	15.85	± .591
		Experiment II				
Fresh	60	5.27	5.33	6.34	5.65	± .104
Same after 3 hours at 100° C.	60	4.47	5.35	6.68	5.85	± .104
2 days old (100 *Colpidia* per cc. introduced)	60	6.19	5.90	7.29	6.46	± .121
Same after 3 hours at 100° C.	60	6.11	5.19	6.88	6.06	± .115
4 days old (100 *Colpidia* per cc. introduced)	60	7.32	7.59	9.33	8.08	± .131
Same after 3 hours at 100° C.	60	7.20	6.43	8.01	7.21	± .116
8 days old (100 *Colpidia* per cc. introduced)	60	9.96	8.33	11.60	9.96	± .194
Same after 3 hours at 100° C.	60	8.27	6.93	9.26	8.15	± .146
		Experiment III				
Fresh	20	.....	.....	.....	5.47	± .172
Same after 1½ hours at 120° C.	20	.....	.....	.....	5.69	± .102
8 days old (100 *Colpidia* per cc. introduced)	20	.....	.....	.....	9.19	± .171
Same after 1½ hours at 120° C.	28	.....	.....	.....	8.41	± .111
		Experiment IV				
Fresh	40	.....	.....	.....	5.63	± .106
Same after 24 hours at 100° C.	36	.....	.....	.....	6.35	± .105
8 days old (100 *Colpidia* per cc. introduced)	40	.....	.....	.....	8.86	± .143
Same after 6 hours at 100° C.	40	.....	.....	.....	10.00	± .133
Same after 12 hours at 100° C.	40	.....	.....	.....	10.1	± .216
Same after 24 hours at 100° C.	28	.....	.....	.....	10.2	± .219

EXPERIMENTS III AND IV

These experiments were performed like the preceding except that 8-day culture fluid was used throughout, and the fluid was heated to a higher temperature in one and longer in the other. The results are presented in Table 6, Experiment III. This shows that for the first division an average of 5.47 hours was required with the *Colpidia* in fresh culture fluid and 5.69 hours in this fluid after it had been autoclaved and that an average of 9.19 hours was required in the old culture fluid and 8.41 hours in the fresh fluid after it had been autoclaved. It indicates, therefore, that, while higher temperature definitely reduces the retarding effect of old culture fluid, it does not eliminate it entirely. These results consequently confirm those obtained in the preceding experiment.

Table 6, Experiment IV, shows that for the first division an average of 5.63 hours was required with *Colpidia* in fresh culture fluid and 6.35 hours after it had been subjected to 100° C. for 24 hours and that an average of 8.86 hours was required after *Colpidia* had grown in it for 8 days and an average of 10, 10.1, and 10.2 hours, respectively, after this fluid had been subjected to heat for 6, 12, and 24 hours, respectively. It shows, therefore, that subjecting old culture fluid for 6 hours or longer to 100° C. increases its retarding effect on the rate of reproduction, and it consequently seems to contradict the results obtained in the preceding experiments. It was, however, observed that after old culture fluid had been subjected to 100° C. for 3 hours it was slightly cloudy and that this cloudiness increased with time, so that after 6 hours or longer, a definite flaky precipitate formed, but that this did not occur in fresh fluid even after 24 hours. In Experiment IV this precipitate was removed by centrifuging before the rate of reproduction was measured. The failure of heat to reduce the retarding effect of the old culture fluid used in this experiment was, doubtless due to chemical changes in the culture fluid associated with the formation of the precipitate and not to lack of destruction of growth substance. The results obtained do not therefore militate against those obtained in the preceding experiments.

The results obtained in the experiments on heat show, then, that the rate of reproduction in old culture fluid is lower than in fresh culture fluid, that it decreases directly with the age of the culture fluid, that subjecting it to 100° C. for 3 hours or to a higher temperature for a shorter time (owing to destruction of growth substance) reduces its retarding effect on the rate of reproduction but not to zero, and that subjecting it to 100° C. for 6 hours or longer increases its retarding effect, owing to profound chemical changes in the culture fluid produced by heat.

SUMMARY

1. In depressions on slides containing 0.1 cc. culture fluid and 1 or more *Colpidia* each, the rate of reproduction increases (as the number per depression increases) to a maximum and then decreases.

2. In depressions containing 1 *Colpidium* and different volumes of culture fluid the rate of reproduction first increases to a maximum (as the volume decreases) and then decreases.

3. In culture fluid which has previously been inhabited by *Colpidia* the rate of reproduction increases to a maximum (as the number of *Colpidia* which had previously inhabited the fluid increases), then decreases.

4. The concentration of available food in the culture fluid can be greatly

changed without any significant change in the rate of reproduction of *Colpidia* in it. This indicates that the reduced rate of reproduction in old culture fluid is not due to insufficient food.

5. If old culture fluid in which the rate of reproduction is below normal is diluted with fresh culture fluid, the rate of reproduction increases (with dilution) to a maximum and then decreases to normal. This indicates that acceleration and retardation in old culture fluid are correlated with the concentration of a substance produced by the *Colpidia*.

6. If culture fluid which has been previously inhabited by *Colpidia* for 2 days is subjected to heat (100° C. for 3 hours), the rate of reproduction in it increases nearly to that which obtains in fresh culture fluid.

7. If culture fluid which has been previously inhabited by *Colpidia* for more than 2 days is subjected to heat, the rate of reproduction of *Colpidia* in it increases somewhat, but never to that which obtains in fresh culture fluid, no matter how long it is heated.

8. If old culture fluid is heated 6 hours or longer a flaky precipitate forms, and if this is removed the rate of reproduction in the fluid decreases. This is owing to chemical changes in the culture fluid caused by heat.

9. It is concluded from the results presented that *C. striatum* produces a substance which alters its growth, accelerating or retarding the rate of reproduction, depending on the concentration of this substance in the fluid.

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PITUITARY AND NERVOUS CONTROL OF PIGMENTARY EFFECTORS, ESPECIALLY XANTHOPHORES, IN KILLIFISH (FUNDULUS)

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THE yellow dermal pigment cells, or xanthophores, of two species of killifishes of the genus *Fundulus* are chromatic effectors subject to humoral control and to a nervous control independent of that of the black pigment cells, or melanophores (Fries, 1931). And there is evidence that they are probably doubly innervated, receiving pigment-dispersing, as well as pigment-concentrating, nerves (Fries, 1942*a*).

The role of the hypophysis in the humoral regulation of the activity of the xanthophores has remained largely a matter of inference from work on other fishes and of analogy with the melanophores, upon which the pigmentary hormone of the hypophyseal pars intermedia is known to exert a subordinate influence in *Fundulus* (Kleinholz, 1935; Abramowitz, 1937*b*). In the minnow *Phoxinus*, Giersberg (1932) could obtain no evidence that the xanthophores have any innervation; he established the pituitary hormone as the dominant factor in their responses. Injections of appropriate pituitary preparations bring about dispersal of their pigment (Abolin, 1925; Hewer, 1926; Giersberg, 1930, 1932; Zondek and Krohn, 1932; Böttger, 1934) and have produced the same result in the xanthophores of various other teleosts, among them *Gobius* (Meyer, 1931), *Macropodus* (Giersberg, 1930), *Serranus*, and *Crenilabrus* (Fleischmann and Kann, 1937). In *Fundulus*, also, pituitary extract disperses the pigment in the xanthophores, according to Matthews (1933) and Abramowitz (1936). Therefore, and although Matthews found hypophysectomy to have no effect on xanthophore response to background colors, it seemed likely that pituitary secretion shared with pigment-motor nerves and their supposed chemical mediators (and possibly still other, undetermined factors) the responsibility for the varying localizations of pigment effected in the xanthophores in the course of the color changes of these killifishes. The following experiments[2] were undertaken to test this inference and, if it were proved correct, to contribute further to an evaluation of pituitary and nervous function in relation to pigmentary co-ordination.

MATERIALS AND METHODS

Fundulus majalis Wahlb. was the chief subject of this study and is the species generally concerned in the following pages, but females of *F. heteroclitus* L. supplied correlative data. Both species were seined the latter half of May in Jamaica Bay, Long Island, and kept in the New York Aquarium's reserve stock tanks (or, for segregation, in small tanks)

[1] All the experiments were carried out at the since dismantled New York Aquarium. For the privilege of using its facilities I owe grateful acknowledgment to Director C. M. Breder, Jr. I am under further obligations to the other officers, R. F. Nigrelli and C. W. Coates, and personnel of the Aquarium for much kind co-operation.

[2] A preliminary report appeared in October, 1942 (Proc. Soc. Exper. Biol. and Med., 51:170–71).

and fed minced clams. The size chosen for experiment was about 6–9 cm. in length.

Experimental colored "backgrounds" were regularly provided by 10-inch culture bowls painted externally (mainly black, white, yellow, or pale blue).[3] Occasionally, for longer adaptation, black- or yellow-painted glass or whitish sand was used as flooring in the aquaria (larger, deeper containers making the fish less irritable than do the bowls). Tiers of overhead windows provided indirect day lighting, at times supplemented by two white-shaded, 75-watt daylight bulbs suspended 5 feet apart and $2\frac{1}{2}$ feet above the table on which the bowls and small tanks rested.

Somewhat dilute running sea water was used, pumped directly from the harbor. Between 13° and 14° C. at the outset, its temperature rose gradually for 6 weeks to 21° and then remained within about a degree of that level for the following, final period of 6 weeks. Occasionally, as for operations, the fish were kept from $\frac{1}{2}$ to 4 hours in standing water, which became warmer (rarely 5°).

All macroscopic determinations of body color were comparative. The test fish or controls or both lots were transferred gently (avoiding excitement) from their previous containers to others, for immediate, simultaneous, direct comparison over the same bottom color (mostly white).

For microscopic inspection of the chromatophores each fish was simply grasped in the fingers (sometimes in wet cotton) and held on an oversized glass stage on a dissecting binocular (with 12.5× oculars and usually 2.3× objectives), so as to bring into view the caudal fin laid flat on its side. Generally, to avoid unnecessary physiologic disturbance, this procedure was brief (totaling often but half a minute, inspection included); and it involved no severe restraint, since the fish usually gave up squirming quickly and did not struggle again for a minute or two.

The fish were iced for most operations. Wet cotton wrapping generally sufficed to check movements that the cold failed to inhibit; this was needed especially when the fish were acclimated to low temperatures.

Hyophysectomy was performed according to the method of Abramowitz (1937*a*), using a trephine and fine-drawn pipette. The completeness of the removal was controlled by examining the tissue sucked out under the binocular; since the compact pituitary in these fishes is of characteristic form, easily seen to be entire or not, further verification, by sectioning, was unnecessary. Apart from one or two prompt losses per dozen operations, the viability of the hypophysectomized fish did not diminish noticeably as compared with nonhypophysectomized: some survived, even through the vicissitudes of further operations, for more than 10 weeks. Infestation by ectoparasitic trematodes and protozoans seemed the chief threat, fatal to normal fish as well as to experimental.

Denervating cuts through the caudal fin or its base were made as earlier described (Fries, 1942*a*). Surprisingly, these little cuts gave rise to pathological symptoms oftener than did the hypophysectomies, presumably because breaking the epidermis of so exposed a part, coincidentally impairing its vasomotor basis for repair and defense, increased sus-

[3] The yellow was the "tulip yellow" speed enamel of Sapolin Company (New York). The pale blue was obtained by mixing Prussian blue into the same company's white enamel until the result appeared equal in shade to the yellow and was approximately equal in effect on the melanophores of killifish kept over it.

ceptibility to infection or vulnerability to molestation by bowl mates.

Implantation of hypophyses into the abdominal cavity was accomplished by means of a fine pipette inserted through a slit in the body wall made just big enough for it. The freshly excised pituitaries were kept in perhaps one hundred times their own volume of Ringer's solution until implanted (mostly within 10–90 minutes); a few drops of the solution were injected at the same time.

RESPONSES TO GROUND COLOR

To determine, first, how closely the local killifishes resembled those of Woods Hole in chromatic reactions, a number of sample lots were tested in the colored bowls. The newly collected fish first changed noticeably within a quarter- or half-minute of transfer to either black or white following a half-hour's adaptation to the other; they passed the mid-point of change in 2 or 3 minutes; and they practically equaled the intense darkness or pronounced pallor of controls in hardly more than 10 minutes. In *F. heteroclitus* (two lots of 11 each at 14° C.) the rate of adaptation to white was considerably faster than the reverse change (in disagreement with the results of Parker and Brower, 1937, and in agreement with those of Cole, 1939, for sea water), but in *F. majalis* (15 and 10, respectively, from two collections, 13 days apart, at 14° and 15° C.) there was no significant difference between the rate of darkening and the rate of blanching.

The macroscopic responses to blue and to yellow conformed essentially to previous findings (Fries, 1931, 1942*a*). There was obvious variability (more than in responses to black and white) in rate of change and in capacity for change. Despite prolonged exposure to the ground-color stimulus, some individuals never became so strongly yellow or bluish as others did sometimes in a few hours. Such change as did occur was, on the whole, markedly slower than the black-white adaptation but began to be distinguishable as a rule within 10, sometimes 4, minutes and usually seemed complete in 3 hours. The initial change toward yellow was at least half again as slow as that from yellow to blue, as determined by several reciprocal tests of *F. majalis* and one of *F. heteroclitus*. The possibility that this difference in rate signifies merely a difference in antecedent production and accumulation of pigment-dispersing or -concentrating substances was not strictly excluded, for the tests were not planned with this point in mind. A striking indication of how far such accumulation may perhaps modify xanthophore responses in *Fundulus* was afforded in an observation that a group of newly yellow-adapted *F. majalis* distinctly lost some of their yellowness in the first 10 seconds of being put for comparison over a white bottom, whereas those long over yellow showed equal change in no less than a minute or more.

The xanthophores of the caudal fin changed somewhat in accord with the color of the bottom; their pigment became concentrated over blue and less so over yellow, but the states assumed by them, on the average, reflected incompletely the fish's dorsal yellowness or non-yellowness, especially in *F. majalis*. In adaptation to yellow and to blue or white, these xanthophores seldom ran three-quarters the gamut of change from fully concentrated pigment to fully dispersed, thus reproducing certain prior results (Fries, 1942*a*); they changed mainly between almost fully concentrated and intermediate or barely more dispersed, even though the fish showed good macroscopic change from bluish to

yellow. In some individuals (distinctly, though not strikingly, yellow-adjusted to the naked eye) the tail-fin xanthophores failed to appear even intermediate after a week in a yellow bowl; in others they combined greater response to yellow with less complete concentration in response to blue. Attempts to induce greater dispersal by substituting orange bowls for yellow proved substantially of no avail. The fact that wide dispersal in the xanthophores of these same individuals appeared occasionally, for unknown reasons, and regularly in consequence of denervating cuts shows that the explanation is to be sought in physiological, rather than morphological, conditions. These might be ionic factors, differences in circulatory supply of hormones or other substances to the fin (since the xanthophores of the scaled region at the base of the fin showed a wider range of change), or a local pattern-like differentiation of pigmentary nerve tone (since xanthophores at the ventral border of the fin were commonly less refractory to dispersal). It is suggestive that, although blue bowls evoked partial pigment dispersal in melanophores, together with concentration in xanthophores, black bowls, while evoking full dispersal in the melanophores, commonly brought about greater dispersal in the caudal xanthophores than occurred in response to yellow.

HYPOPHYSECTOMY

Fundulus majalis.—Four lots were hypophysectomized at various intervals, and 54 fish survived for determination of their responses to different-colored bottoms at least 5 days after the operation, presumably amply long, that is, for exhaustion of pituitary substances already in the blood (Parker, 1940*c*, 1940*d*). On the first two occasions blank operations (the entire procedure except sucking out the pituitary) were also performed on numbers roughly equaling the two hypophysectomized lots (a little more than half the total), to provide controls additional to intact animals; but thereafter, since their chromatic reactions were evidently identical to those of the normal controls, blank operations were omitted.

The first observations on these fish gave support to the view, based hitherto as published on only *F. heteroclitus*, that the pigmentary hormone of the pituitary is, at most, a minor accessory factor for normal dispersal in the melanophores of *Fundulus* (cf. Parker's review, 1940*a*): the hypophysectomized fish darkened and paled over black and over white, respectively, about as fast and as fully as those having pituitaries.

The subsequent observations disclosed different circumstances as regards response to yellow and to blue or white. The hypophysectomized resembled the control fish after a day or more in blue or in white bowls. But fish lacking their pituitaries were conspicuously incapable of responding normally to yellow, irrespective of the duration of their stay over that color. This fact was evident in all cases; but, after establishing it in the earlier determinations, I directed my attention more particularly to the question of whether the hypophysectomized fish retained any limited power to adapt to yellow at all. It became clear that they did. When half of one batch of fish hypophysectomized 11 days before, and last kept over yellow, were compared (simultaneously over white, as usual) with the other half that had been kept over blue, the former were certainly, although only slightly, more yellowish. And after the two sets had had their bowls interchanged, those previously more bluish became certainly more yellowish, while

the others lost their yellowishness. Other reciprocal tests of this sort, in which white bowls replaced the blue, showed that hypophysectomized fish became definitely yellower over yellow than over white. Several times the difference grew distinguishable in 10–15 minutes.

On microscopic examination the xanthophores of the tail fin after hypophysectomy displayed mainly the concentrated state. Short or long stimulation from a yellow bottom failed to induce even the limited dispersal of their pigment, such as was usual in the normal fish. In view of the characteristically narrow range of participation by the tail-fin xanthophores in adaptive changes by the intact fish, it is not surprising that in the hypophysectomized fish the caudal xanthophores did not convincingly disclose changes corresponding to the slight adaptive yellowing and de-yellowing observed with the naked eye. Does this mean that xanthophore changes had no share in effecting those macroscopically visible responses, or, in other words, that the differences in yellowness depended exclusively upon the activity of other chromatophores? That is unlikely, for various reasons. The leucophores, or guanophores, appear to take no such active part in the responses to yellow and blue as in those to black and white (Fries, 1942*b*). The melanophores, if differing at all in their responses to yellow and to white, tend to concentrate their pigment and thus to unmask deeper xanthophores, more completely when over white than when over yellow. It is particularly pertinent to note, moreover, that even in the tail fin the xanthophores did show definitely distinguishable dispersal of their pigment in response to black as the bottom color, and also, on several occasions, in apparent consequence of handling and holding the fish on the stage for half a minute, and sometimes, too, after injection of adrenalin.

It was therefore clear that removal of the pituitary, though interfering greatly, had not deprived the xanthophores of all capacity for pigment movement.

Fundulus heteroclitus.—Among two lots, 20 fish were available for tests 5 or more days after hypophysectomy. Four other fish which survived blank operations equally long showed no differences from normals; so thereafter only normals were generally used as controls.

The loss of the pituitary in this species, as in the other, did not seem to interfere with the responses to black and to white (by the melanophores) but did prevent normal adaptation to yellow. In fact, all the hypophysectomized fish displayed unwonted uniformity of coloration over a pale ground, regardless of its color and of how long they were kept over that color; they were grayish, resembling white-adapted and blue-adapted controls, but were not quite so pale as the former might be,[4] or with the same glint as the latter. Comparison of hypophysectomized fish over yellow with others kept over blue and others over white failed to disclose any sure differentiation between them, such as appeared in pituitaryless *F. majalis*. The caudal xanthophores in hypophysectomized *F. heteroclitus* from yellow had their pigment largely concentrated (virtually as much as in normal controls from blue) and clearly contrasting with the moderate dispersal in controls from yellow. This dependence of yellow-adaptation and of the response of xanthophores to yellow upon pituitary function was contrary to results of hypophysectomy reported by Matthews (1933). Yet the xanthophores

[4] This brings to mind the pigment-concentrating "W" hormone ascribed to the pituitary (cf. Waring, 1940, and Healey, 1940).

in this species did remain in some measure able to accomplish dispersal of their pigment even in the absence of hypophyseal influence, for, several times and in several individuals, handling and holding under microscopic inspection plainly resulted in dispersal in the xanthophores as well as in the melanophores (as compared with a band of denervated xanthophores and melanophores in the same fish which did not show dispersal). Such changes, however, signify little concerning what factors regulate response to "background" and so are inadequate to confirm Matthews' conclusion as to the dispensability of the pituitary.

PERIPHERAL DENERVATION IN RELATION TO HYPOPHYSECTOMY

The clearly shown interference by hypophysectomy with normal yellow-adaptation and pigment dispersal in the xanthophores, which persisted all the weeks that the pituitaryless fish were under observation, called for a reconsideration of the question of nervous control of xanthophore responses. The experiments reported in this section were directed to this purpose.

Fundulus majalis.—Eleven or more days after removing the pituitaries, and over a period of 2 months, I made cuts transecting two or three basal fin rays in the tails of four groups totaling 45 fish, of which a third were females. Nearly half these cuts and some later one-ray cuts were made just distal in location to the scale-covered region (conforming to the original method of Wyman, 1924); the rest were proximal to the basal blood-vessel arc in the tail and at no time interrupted the circulation (Fries, 1931, 1942*a*). Without exception the caudal band, thus denervated, turned both darker and yellower because of the dispersing of the pigment in the chromatophores situated in the band, environmental conditions being so adjusted as to keep the chromatophores in a more concentrated state elsewhere in the fin. In this result the hypophysectomized differed in no wise from normal fish, excepting that the band in the hypophysectomized may have taken a few more minutes to effect the change and that the dispersal in it tended not to go to a fully equal extreme, especially in the xanthophores, which recalls the reduced melanin dispersal found in new bands in hypophysectomized catfish (Parker, 1940*d*). Evidently in this teleost, contrary to Vilter's (1939) hypothesis based on other species, the pigment dispersal that results from cutting the nerves leading to the chromatophores depends neither upon the continued functioning of the hypophysis nor upon the presence of its pigmentary hormone.

In subsequent behavior the bands did not differ essentially from those of normal fish. They gradually faded, as their xanthophores and melanophores assumed within a few days the more concentrated state characterizing the chromatophores of the intact sectors of the tail. They took less time to fade in fish kept over blue or white bottoms than in fish over other colors. They faded sooner if narrower than if wider; also, sooner in the margins than in the axis of the band. Sometimes, while still relatively dark and yellow, the bands faded quickly as the fish was handled or held on the stage of the microscope, just as noted often in those with the pituitary intact (Fries, 1942*a*). When fish with week-old or fortnight-old faded bands were transferred to black bowls, the bands failed to keep pace with the change that occurred in the other sectors, where the pigment became more dispersed; such limited dispersal as occurred in the bands lagged, and the lagging was clearer in the axis of the band

than in its margins. The failure of the melanophore and xanthophore pigment to disperse in the band as much as elsewhere, or as soon axially as in the margins, was observed also in cases of the pigment dispersal that occurred as a consequence of holding the fish. In the absence of strong redispersal in the old faded bands there was scant opportunity for them to lag in a corresponding manner when an opposite response was induced in the innervated regions by transferring the fish to blue bowls; when pigment concentration did occur more or less distinctly in the innervated xanthophores, which also seldom had much scope for change in that direction, no lagging in like response was discerned in the band.

It was apparent, then, that, although the pituitary is essential for normal participation by the xanthophores in color change, particularly in adaptation to a yellow ground color, it is possible even in killifish deprived of pituitary secretion to reproduce certain parts of the evidence held to signify that humorally mediated pigment-dispersing, as well as pigment-concentrating, nerves intervene in the control of xanthophore response (Fries, 1942*a*). In order to enlarge upon this evidence or to cast new light on the question, I repeated an experiment previously reported for nonhypophysectomized fish, involving flanking and control secondary denervated bands (Fries, 1942*a*). Eight hypophysectomized females were available with fully faded primary bands that had been produced by cutting three fin rays just anteriorly to the transradial arc of blood vessels. When the dark and yellow secondary bands, produced 17 days after the primary cut in 6 of the fish, had been established 5–30 hours, the same effect as in the earlier, nonhypophysectomized *Fundulus* became apparent: the innervated control band showed no change, being sharply demarcated from the adjacent secondary bands, while the distal part of the primary band, especially along the margins adjoining the secondary bands, showed some pigment dispersal by the xanthophores plainly in 2 and faintly in 3 of the 6 fish. The experiment on the final 2 fish, which were given the secondary cuts 20 days after the primary (when nerve regeneration had at most begun in the proximal end of the primary band in one of the two, as revealed by transfer to black), yielded similar, passably distinct, affirmative results. Allowing for the somewhat lesser yellowness of the secondary bands in these hypophysectomized fish, one may conclude that the affirmative indications of a spreading of the pigment dispersal from the newly denervated zones into the old occurred fully in proportion to their previously found frequency in the presence of the pituitary (Fries, 1942*a*). Consequently, whether or not they signify diffusion of neurohumoral substance from dispersing pigmentary nerves, as seems probable, they must at least be caused by something other than a pituitary hormone.

Fundulus heteroclitus.—Caudal denervating cuts, done 4 days after hypophysectomy in 13 fish and a month after it in 6 (with the water at 21° C. for both lots), gave rise to less uniform results regarding the xanthophores in this species. Although the bands darkened through melanophore change regularly and promptly, xanthophore pigment dispersal occurred in only 3 of the group of 6, in which the cuts transected two rays just distad of the scales, interrupting the radial blood vessels. This is a lower ratio than I found in my first work on this killifish (Fries, 1931); but, since an equally low ratio of yellow bands resulted from identical operations carried out the same

day on 7 control fish, it did not appear to be due to the loss of the pituitary. The other lot was more curious. Although the cuts, which transected three rays proximally, this time avoided interruption of the blood supply, at most 3 of the 13 fish showed any yellowing of the band discernible within the usually sufficient period of the first 20 minutes. In fact, the xanthophores of the band in the other 10 assumed a state for the most part obviously more concentrated than before and contrasting especially with the xanthophores outside the band, which exhibited the quick pigment-dispersing reaction to the handling involved in the examination. And next day only 1 of the 13 bands was relatively yellow when first inspected. But reinspection some hours later and the following day disclosed more cases of dispersal of the xanthophore pigment in the bands, so that altogether 9 of these 13 fish eventually displayed yellow bands (on the fifth or sixth day after hypophysectomy).

Thus, in this species, too, the pituitary proved to be not essential for the yellowing, as well as darkening, that characteristically occurs in regions subjected to denervating cuts.

Hypophysectomized *F. heteroclitus* afforded other observations, duplicating some made on *F. majalis*. Quick temporary fading of denervated bands occurred in consequence of handling, and transitional pigment states (suggesting axipetal change due to neurohumoral invasion) appeared in the margins of bands that showed pigmentary contrast with neighboring sectors. Clearly, for such chromatophore behavior, also, the pituitary factor is not indispensable.

IMPLANTATION OF HYPOPHYSES AFTER HYPOPHYSECTOMY

Removal of the pituitary might owe its observed effect to coincident damage done to the nervous system rather than to interruption of pituitary endocrine secretion. To test this point I implanted whole pituitaries of *Fundulus* intraperitoneally, from 3 to 6 each, in 7 or 8 individuals of each of the two species, a few of them as soon as 5 days and some as late as 2 months after they were hypophysectomized. All[5] thereupon turned yellow when kept over a yellow bottom, some fully as much as the yellowest normal controls. Unlike *Phoxinus* under similar treatment, as reported by Giersberg (1932), all were less yellow after a stay over white or blue, though they were still distinguishable, as rather yellowish, from pituitaryless fish and from normal controls kept likewise over white or blue. On transfer from the white or blue to yellow, or vice versa, they became yellower, or less yellow, respectively. This change became visible within half an hour of transfer in either direction. A slight change *from* yellow-adaptation appeared to occur (as already noted for normal fish) after only a few seconds in a white comparison dish, whereas the complete change *to* yellow took a few hours. While the melanophore pigment remained fully concentrated in the caudal innervated regions in case of any pale ground color and was well dispersed in the denervated bands irrespective of the ground color, the xanthophore pigment in the innervated regions changed in a sense corresponding to the naked-eye effect, being more or less widely dispersed in the yellower fish and less so, though not completely concentrated, in the only slightly yellowish ones kept over blue. In the latter the innervated regions contrasted with denervated bands, even as regards the xanthophores. The bands, whether newly cut or old and previously faded,

[5] Except one which had received only two pituitaries and which, in addition, died in 2 days from a dermal infection.

were yellow as well as dark, except temporarily during apparent reaction to handling or holding at the microscope. (The latter reaction involved fading of the band in about 2 minutes, as in nonimplanted fish; following it, redispersal of the pigment in the band independently of the other parts became pronounced in about 20 minutes after the fish got back to its blue dish.) New-cut bands failed to fade in response to a blue or white bottom in the few days that sufficed for such fading response to be manifested in nonimplanted controls. Also, the pigment-concentrating reaction to cutting that is reported for 10 *F. heteroclitus* in the previous section did not appear in any of 3 *F. heteroclitus* given the tail cut after the implantation.

The effects of the implantations diminished gradually, to become indistinguishable in a week or two.[6] Reimplantations into 2 or more individuals of each species resulted, without exception, in renewing their yellowness.

Plainly, pigmentary nervous function had not been irreparably impaired by hypophysectomy. The implantation of pituitaries supplied a humoral substance without enough of which the innervated xanthophores could not make the maximum response to yellow ground and with so much of which they could not concentrate their pigment completely. In the denervated xanthophores the pigment became dispersed and remained so as long as the supply of pituitary substance was maintained, except when the influence of this supply, doubtless relatively constant, was temporarily overcome by some opposing humoral factor.

Implantation of 3 *F. majalis* pituitaries each in 3 *F. heteroclitus* seemed to exert fully as stong a yellowing influence as 5 *F. heteroclitus* or 4 *F. majalis* pituitaries each in some *F. majalis*. These meager quasi-quantitative data warrant at most a suspicion or two concerning, for example, a possibly greater sensitivity of the xanthophores in *F. heteroclitus* to the pituitary factor as compared with *F. majalis*, a difference which might be related to the completer inability of the former species to respond to yellow when hypophysectomized.

[6] This result, suggesting gradual resorption, is consistent with the histological and other data of Emery (Anat. Rec., **66**:253, 1936) and Matthews (Biol. Bull., **79**:207, 1940) on the survival of such implants.

DISCUSSION

In a review of the foregoing results the following points emerge as among the most salient. (1) The pituitary is necessary for normal adaptive color change, i.e., dispersal of pigment in xanthophores, in response to a yellow bottom. But its absence does not deprive the xanthophores of all capacity or even of all color-adaptational capacity for dispersing their pigment or for concentrating it again. (2) The pituitary is not essential for the yellowing of caudal bands that results from denervating cuts, or for their subsequent fading. But, when present, a humoral substance that it supplies causes pigment dispersal in such bands and opposes pigment concentration on the part of the xanthophores of innervated zones in response to a white or blue bottom. (3) When the supply of pituitary substance is centrally unmodifiable (as when derived from implants) and not too great, the xanthophores of *innervated* regions still effect some degree of adaptation to the ground color (showing relative concentration over white or blue versus relative dispersal over yellow). (4) When deprived of the pituitary substance, the xanthophores of *denervated* bands still behave distinctively; for example, they fail to keep pace with the adaptive changes of the xanthophores of innervated sectors and sometimes react

to handling by quick, contrasting pigment concentration.

A first conclusion follows obviously: a pigment-dispersing secretion from the pituitary is one, but only one, of at least three factors concerned in controlling xanthophore changes. Presumably this pituitary substance is the melanophore-"expanding" and erythrophore-"expanding" hormone, intermedin (Zondek and Krohn, 1932); according to Abramowitz (1940), the present consensus favors a single intermediate-lobe hormone for all the chromatophores. Incidentally, its effectiveness on denervated xanthophores, even when the nerves have doubtless fully degenerated (over 2 weeks after cutting), substantiates the view that it must exert a direct action on the cell, in the case of the yellow chromatophore as well as the red and the black (cf. Giersberg, 1934, and Böttger, 1934, for erythrophores; Parker, 1940*c*, for teleostean melanophores).

Secondly, a former conclusion is confirmed: the xanthophores have direct pigment-motor innervation (Fries, 1931). What the exact nature of this innervation may be invites closer scrutiny of available data.

There is general agreement that, wherever chromatophores receive any innervation, it includes pigment-concentrating nerves. That this holds true for killifish xanthophores seems clearly established. Upon the excitation of such nerves must depend not only the pigment concentration that innervated xanthophores may show in contrast to those of denervated bands even in the continued absence of the pituitary-dispersing factor but still more essentially the ability of innervated xanthophores to overcome the dispersing influence of moderate amounts of that factor (from the implanted pituitaries) and so to concentrate their pigment, as the denervated xanthophores cannot, in adaptation to blue.

There is less agreement about whether pigmentary innervation may include also pigment-dispersing nerves. But recently Parker and Rosenblueth (1941) have supplied more direct evidence, than previously adduced, in fresh support of the hypothesis of double innervation of teleost chromatophores; and Parker has extended its corollary buttresses in later contributions (1941*b*, 1941*c*). That the results of denervating cuts following hypophysectomy do not impair the case lately presented (Fries, 1942*a*) for applying the hypothesis to the xanthophores of *Fundulus* is perhaps manifest in the details concerning denervated bands recounted in the foregoing pages, especially to those familiar with the neurohumoral explanation of chromatophore activation. The pertinence of a few of the points may, however, be noted here. (1) The quicker and greater change by innervated xanthophores than by denervated xanthophores in cases of pigment dispersal (response to black or to handling) after hypophysectomy seems to require the action of dispersing nerves. (2) If such nerves do not exist and so cannot be excited by the denervating cut, then how shall we account for the pigment dispersal that occurs (irrespective of the bottom color) in the newly denervated band even in the absence of the pituitary's dispersing factor? The long-familiar alternative explanation, namely, that the chromatophores assume a putative state of rest, with their pigment dispersed (cf. Parker, 1940*b;* Parker and Rosenblueth, 1941), when released by a denervating cut from the concentrating influence of nerves, would seem to lose force *pari passu* with the later, characteristic, grad-

ual fading of the band, as in white-adapted fish. Does this perhaps leave those who reject the hypothesis of dispersing innervation in the position either of ignoring a phenomenon repeatedly stressed (for nearly a century) as relevant or else of needing to muster some *ad hoc* explanation for the subsequent fading (qua chromatophores' abandonment of rest) that undeniably occurs despite continued default of both nerve stimulation and any possible concentrating hormone from the pituitary? Among those who have stressed the possibly misleading vascular consequences of cuts that unquestionably sever vasomotor nerves (along with any others), if not also blood vessels, Waring (1940) remarks that vasoconstriction favors pigment concentration by reducing the supply of pituitary dispersing substance. As regards the present results, however, it is difficult to conceive how this or any other interpretation based on vasomotor action or paralysis could account for *both* the early dispersing and the later slow concentrating of the pigment in the xanthophores and the melanophores in denervated bands of white-adapted killifish deprived many days or weeks of their pituitaries. (3) In the experiment with secondary denervated bands, any residue of the pituitary hormone could hardly be effective 12–16 days after hypophysectomy—least of all selectively so in the distal part of a many-days-faded primary band. This invalidates resort to the pituitary to explain the observed redispersal of pigment in the primary band where flanked by the new yellow secondary bands; and it reinforces the conclusion (Fries, 1942*a*) that such an effect is probably the result of a diffusive invasion of a pigment-dispersing neurohumor secreted by dispersing nerve endings to the xanthophores in the secondary bands because of the excitation occasioned by cutting the nerves.[7]

Now, if double innervation of chromatophores, including xanthophores in *Fundulus*, be admitted, the initial pigment-concentrating reaction to caudal cuts, as reported for *F. heteroclitus*, becomes perhaps less anomalous than it seems at first sight. For, to account for it, one might then assume that normal variation in detailed nerve supply tends in this species, oftener than in others studied, to permit initial dominance of the concentrating fibers and their neurohumor over the neurohumor from the dispersing fibers, which are generally more lastingly excited by the injury of cutting (Parker, 1941*a*). Incidentally, such an assumed tendency to dominance of concentrating over dispersing nerves and neurohumors is not inconsistent with the inability of *F. herteroclitus* to respond noticeably to yellow unless supplied with the pituitary factor. That the controlling mechanisms of pigmentary effectors do vary both as between teleostean species and as between the types of chromatophores needs nowadays no special proof.

Neither nerves nor pituitary secretion can account for the quick disappearances of the deeper color of denervated bands in apparent reaction to handling. Diffusion of concentrating lipohumor from the innervated regions would necessarily be

[7] In a valuable review which came to my notice only after the completion of this paper, Waring (1942), who appears not to have had access to Parker's work published in 1941, marshals criticisms of the hypothesis of double innervation. His uncompromising stand that "the *only* [my emphasis] acceptable evidence for direct nervous control is *reversible* response of effectors to stimulation after stoppage of the circulation" would seem suited to perpetuating that divergence of views between the "Hogben and Parker schools" which results partly, he observes in other connections, from neglect of different classes of experiment. This is not to disdain the evidence he requires, should it be found available.

too slow. Therefore, the quick fading of the bands must be attributed to temporary access of some blood-borne substance. That this fading occurred jointly with temporary reduction or stoppage of blood flow in the tail fin, just as previously described for unhypophysectomized fish (Fries, 1942*a*), was many times confirmed in hypophysectomized *F. majalis*. Attempts to verify a thus renewed suspicion that the substance might be adrenaline were inconclusive. In some hypophysectomized *F. majalis*, yellow denervated bands faded after intraperitoneal injection of 0.03–0.2 ml. of commercial adrenalin solution, as found by Abramowitz (1936). In several, such injection resulted in pigment dispersal in the innervated xanthophores, sometimes contrasting with an opposite reaction of the denervated xanthophores.[8] Altogether, as regards the xanthophores of both species, the results of these adrenalin injections were conflicting and could best serve as simply a start toward a more thoroughgoing study of the perhaps complex chromatic role of the adrenal secretion in killifishes.

To sum up, I conclude that the xanthophore-controlling mechanisms in *Fundulus* probably include: (1) both concentrating and dispersing pigment-motor nerves and their respective neurohumors, or chemical mediators; (2) a pigment-dispersing pituitary substance, presumably intermedin, which is more important for color-change response to a yellow ground than the dispersing nerves and neurohumor and which is more essential for xanthophore dispersal in *F. heteroclitus* than in *F. majalis*;[9] and (3) an unidentified pigment-concentrating, blood-soluble substance (perhaps adrenaline). This scheme of co-ordinating devices resembles that with which the melanophores of *Fundulus* are probably provided, though there is a marked difference in the relative need for the pituitary substance in pigment dispersal, the melanophores being dominated by nerves and hardly affected by its absence. There is likewise some obvious similarity to the efferent part of the nervous and humoral reflex system controlling the melanophores in the catfish (Parker, 1940*c*); the differences lie less in the pattern of the system than in chromatophore reactiveness or in the relative dominance of the components of the system. Chromatically *Fundulus* is, as Giersberg (1932) judged, intermediate between dominantly nerve-controlled types and primarily hormone-regulated types of teleosts.

These conclusions give new insistence to the question, argued by Abramowitz (1936), whether or not the neurohumors from the melanophore nerves are identical with those from the xanthophore nerves. Abramowitz concluded, with a reservation, that they are not. My observation that the caudal xanthophores in normal and hypophysectomized *Fundulus* were more able to disperse their pigment in response to black (when the melanophores dispersed theirs) than to yellow (when the melanophores kept theirs concentrated) does not confirm that conclusion. Yet my results in general are hardly such as to disprove it. Rather are they an incentive to a new attack on the question of the precise means of control, presumably central in origin, of the independent responses of different types of

[8] The melanophores reacted to the adrenalin in the usual way. Both innervated and denervated regularly concentrated their pigment. The former effected redispersal sooner (in response to black).

[9] It may be significant that *F. majalis* is the more "nervous" type, of slenderer build and livelier habits.

chromatophores—a question made the more pointed by the possibility that the xanthophore nerves may prove to be adrenergic and cholinergic, just as the melanophore nerves in *Fundulus* may be, to judge from the catfish (cf. Parker, 1940*c*, 1941*a*). Differences between the xanthophores and melanophores in specific reactivity to intermedin and to other substances may not suffice to provide the chromatophores with the independence of response that many investigators have verified as characteristic in various teleosts.

SUMMARY

Facts.—Fifty-four *F. majalis* and 20 *F. heteroclitus* survived hypophysectomy for study of their chromatic responses 5 or more days later. Hypophysectomy abolished normal adaptive color change in response to a yellow bottom, but *F. majalis* still showed a limited response. While hardly affecting innervated melanophores, lack of the pituitary prevented normal pigment-dispersing response to yellow by innervated xanthophores, though these xanthophores still accomplished dispersal under certain exceptional conditions and, at least in *F. majalis*, also in response to black, and they could then concentrate their pigment again.

One or more caudal fin rays were transected in 45 of these *F. majalis* and 19 of the *F. heteroclitus*, mostly 11 or more days after the hypophysectomy. In the caudal bands thus denervated, the yellowing and darkening that characteristically results from such cuts was not prevented or radically hindered by the lack of the pituitary. The bands resembled those of unhypophysectomized *Fundulus* also in their subsequent fading, in re-yellowing (as when flanked by new yellow bands), and in other changes.

Intraperitoneal implantation of fresh pituitaries (3–6 per recipient) into 7 or 8 of each of these sets of killifishes, between 5 days and 2 months after the hypophysectomy, induced pigment dispersal in denervated chromatophores of faded bands, regardless of the ground color. In innervated xanthophores it made possible typical dispersal in response to yellow and opposed, without abolishing, pigment concentration in response to white or blue.

Conclusions.—These facts and the detailed evidence provide (1) proof that a pigment-dispersing pituitary secretion, presumably intermedin, is one of several factors controlling xanthophore changes in *Fundulus* (it acts directly on the cell); (2) confirmation that the xanthophores are supplied with pigment-motor nerves, including pigment-concentrating fibers; (3) added support for the hypothesis that the xanthophore (also melanophore) innervation further includes pigment-dispersing nerves, chemically mediated by a corresponding neurohumor; and (4) strengthened indication that xanthophore regulation involves a blood-borne, nonpituitary, pigment-concentrating substance (adrenaline?). In scheme, this system of co-ordinating devices resembles that governing the melanophores in *Fundulus*. The melanophore system differs primarily in the more complete dominance of the nerves, the pituitary being hardly needed for pigment dispersal.

Xanthophore regulation is not identical in the two species of *Fundulus*. The pituitary mechanism predominates in *F. heteroclitus;* the nervous is relatively important in the livelier species, *F. majalis*.

The nonidentity of the xanthophore and melanophore neurohumors is questioned.

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FEATURES OF THE ELECTRICAL RESPONSES OF THE BEE-MOTH EYE

(Seven figures)

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THE electrical responses of the compound eye of the bee moth (*Galleria mellonella*) to illumination have been investigated with the eyes completely dark adapted or light adapted to various degrees. Jahn and Crescitelli (1939, 1940) have investigated the electrical responses of the eyes of another species of moth (*Samia cecropia*), but the present work reveals that the bee-moth eye differs significantly from that of the Cecropia moth as regards certain noteworthy features of the electrical complex. However, the general nature of the response of the bee-moth eye is of the "night" type described by Jahn and Crescitelli (1940) for certain other species of insects, including the Cecropia moth.

Granit (1933), as a result of his analysis of the electrical responses of the cat's eye, proposed the idea that three components in the eye are responsible for all of the various waves in the cat's electroretinogram. Some workers have attempted to apply this analysis to arthropod eyes; but the present study indicates that Granit's three-component theory may not be applied, at least without modification, in interpreting the electrical behavior of the compound eye of the bee moth.

MATERIALS AND METHODS

The animals used in these experiments were moths that had emerged from one to two days previously and were taken from cultures raised at 30° ± 0.2° C. Approximately equal numbers of animals of both sexes were used, but no consistent sex difference was observed in the recorded potentials.

The lead-off electrodes from both eyes were applied according to the method described by Jahn and Crescitelli (1938), each animal being mounted on a wax block in a light-proof compartment within an electrically shielded thermostat maintained at 25° ± 1.0° C. In each case, one eye was kept in the dark and the entire cornea of the other was illuminated as desired, the light being furnished by a 500-watt projection bulb (110-volt, D.C.) placed 45 cm. from the eye. To avoid a temperature increase during long exposures, the light, before reaching the eye, was passed through 5 cm. of a 1 per cent $CuCl_2$ solution to remove the heat rays. Such a filter has been found by Coblentz (1911) to remove practically all infra-red radiations while transmitting about 70–80 per cent of the visible rays. The illumination at the surface of the eye was close to 290 foot-candles.

The duration of the short exposures was controlled by means of a combination of rotary and manual (Kodak) shutters. The rotary shutter, consisting essentially of a black cardboard disk with the proper sector cut out, was driven by a small phonograph turntable motor stroboscopically adjusted to the desired speed. The manual shutter was used to control the number of exposures given at

a time by the rotary shutter. Exposures of 1.0 second or longer were given through the use of the manual shutter alone. In all cases the duration of the exposure was recorded by directing part of the stimulating beam of light onto the photographic paper by means of a system of small mirrors, a slit, and lens.

The potentials were recorded through the use of an Einthoven string galvanometer (Cambridge Instrument Co.) pre-

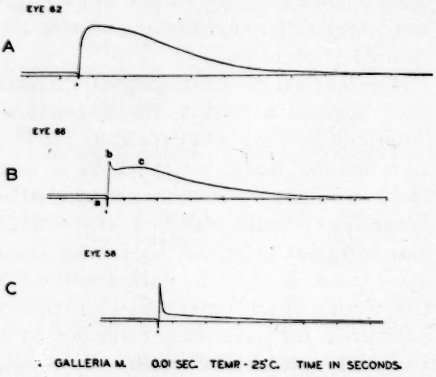


FIG. 1.—Responses from three different eyes (completely dark adapted) to 0.01-second exposures. Response at *C* is from a "fast" eye. Mark below each record indicates the exposure.

ceded by a vacuum-tube amplifier especially designed by Forbes and Grass (1937) for use with the string galvanometer. The direct-coupling circuit in the amplifier was employed in all cases, permitting the accurate recording of long, slow potential changes as well as rapid fluctuations. This amplifier is provided with a suitable calibrating system.

In the evening the animals were prepared for use as mentioned above, given preliminary tests, and kept in complete darkness, so as to be ready for the experiments on the following day. This insured that each eye was completely dark adapted at the start of the experiment. With the eye in this condition a 0.002- or 0.01-second exposure was given to obtain a response that could be used as a standard during the experiment. Whenever necessary, the standard exposure was repeated to check the completeness of dark adaptation.

All records of the electrical responses presented here have been traced from the originals to facilitate reproduction. An upward deflection indicates increased negativity at the cornea of the illuminated eye, the recording being arranged in this way as an aid in making comparisons with the vertebrate electroretinogram as conventionally recorded.

RESULTS

In this study a relatively complete series, comprising over twelve hundred photographic records, were obtained from 36 adult moths. The response obtained from the eye of *G. mellonella* is found to be very similar in wave form to that obtained from the vertebrate retina; but, as is to be expected, all components are opposite in sign. For short flashes on the completely dark-adapted eye, it begins with a fast, positive *a*-wave (downward deflection) after a latent period of 10–15 sigma. This is followed by a *b*- and a *c*-wave more or less completely fused and negative in sign (upward deflection). In Figure 1, *A* and *B* show two such responses to single 0.01-second flashes, the response in *B* exhibiting incompletely fused *b*- and *c*-waves. The negativity of the *c*-wave may last from 5 to 10 or more seconds after such an exposure—a fact that has not been clearly noted in previous work on arthropods. This slow wave of negativity is quite comparable to the positive *c*-wave found in the vertebrate electroretinogram by many workers. The amplitude of the fused *b*- and *c*-waves increases with the duration of the stimulus up to an exposure of about

1.0 second. For longer exposures than this, the response curve, after the peak has been reached, drops very slowly toward the base line for the remaining duration of the stimulation (Fig. 2, *E*). However, it always remains well above the original base line, even for exposures up to 30 seconds, the longest used in this study. In Figure 1, *C* shows a response to an exposure similar to those given in

With the eye dark adapted and exposures lasting longer than the time required for the *b*-wave to reach its maximum, the "off" of the stimulating light causes an immediate sharp drop toward the base line. This may be followed by a continuation of the *c*-wave when the exposure has a duration of about 0.1 or 0.2 second or by a gradual descent toward the base line for exposures of 1.0

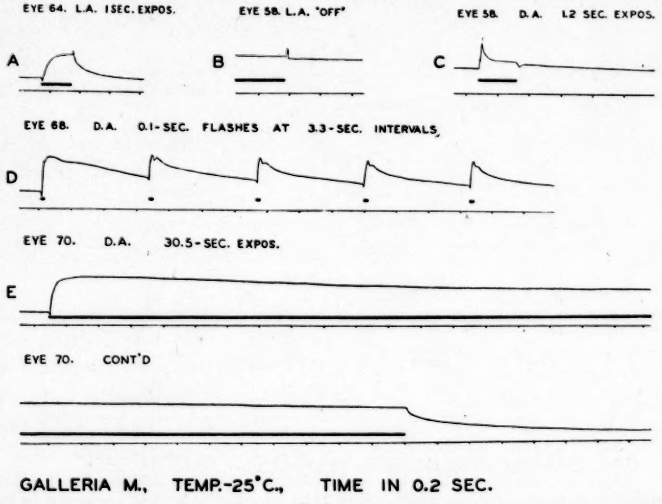


FIG. 2.—Records showing various types of responses at the cessation of stimulation: *A*, response to a 1-second flash given 30 seconds after a 20-minute period of light adaptation; *B*, off-effect of a "fast" eye after 20 minutes of light adaptation; *C*, response to a 1-second flash from a completely dark-adapted "fast" eye; *D*, responses to a series of 0.1-second flashes given at 3.3-second intervals; *E*, response to a 30-second exposure from a completely dark-adapted eye.

records *A* and *B* (Fig. 1) obtained from other eyes, but the *c*-wave appears to be almost completely lacking in this case. Five "fast" eyes, which gave the type of response shown in *C*, were found among the animals studied. The rapid drop of the response to the base line, after the *b*-wave, was characteristic of these eyes for all durations of stimulation and degrees of adaptation; but, as yet, no logical explanation can be offered for this behavior, as the eyes responded normally in all other respects.

second or longer, as in Figure 2, *E*, in which the response at the "off" is still typical of the dark-adapted eye even after an exposure of 30.5 seconds.

Light adaptation (20 minutes at an intensity of 290 foot-candles) causes a marked reduction in the amplitude of the *a*-, *b*-, and *c*-waves and in the duration of the total electrical response, the reduction in the duration of the response being particularly marked with relatively short flashes. This can be seen in Figure 3 by comparing the response of the complete-

ly dark-adapted eye with that of the same eye after only 0.5, 1.0, or 2.0 minutes, etc., of dark adaptation, i.e., while the eye is in a relatively light-adapted state. The early, rather rapid return to the base line appears to be largely due to the absence or marked reduction of the *c*-wave in the response of the eye that is light adapted to a considerable degree.

set of the *d*-wave remains practically constant during the period of extinction of the *d*-wave accompanying progressive dark adaptation, and the final drop to the base line is smooth in all cases.

In comparing the responses of the dark- and light-adapted eyes at the cessation of stimulation, it appears that a different component is acting in each case. In the dark-adapted eye the first

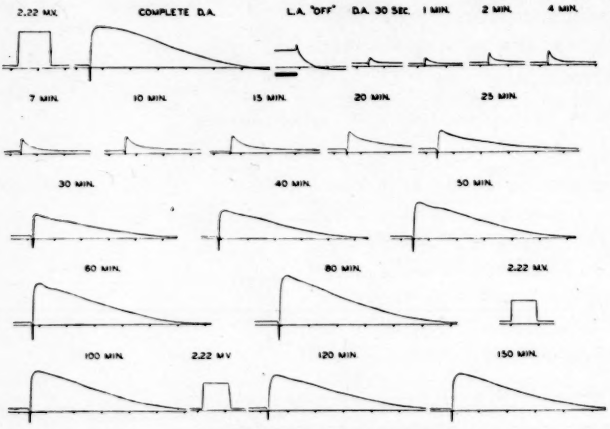


FIG. 3.—Series of 0.01-second flashes given before light adaptation and at various intervals after light adaptation to follow the course of progressive dark adaptation. Time of dark adaptation printed above each record.

A further characteristic of the light-adapted eye is the appearance, in response to exposures of 1-second duration or longer, of a definite negative deflection or *d*-wave at the "off" of the stimulating light. The amplitude of this wave may reach 0.35 millivolt after 20 minutes of light adaptation, and it gradually diminishes as the eye becomes progressively dark adapted. Figure 2, *A*, shows the nature of the response to a 1.0-second flash given 30 seconds after a 20-minute period of light adaptation, the *d*-wave being distinct. The time interval between the end of stimulation and the on-response is a sharp downward deflection. That this is not merely a phase of the passive return to the base line may be seen in Figure 2, *C* and *D*. In record *C* the off-effect of a dark-adapted "fast" eye is shown. In this case the absence of a substantial *c*-wave has left the response very near to the base line, so that the downward deflection causes a notch followed by a rebound. In Figure 2, *D*, the off-effects of a series of 0.1-second flashes are superimposed on the *c*-wave at an early stage in its development, the eye being initially in the completely dark-adapted state. The eye remains essen-

tially dark adapted throughout such a series, each flash being brief and the interval between flashes long. The second response in this series shows particularly well that there is an active factor involved at the "off." The response curve is forced sharply downward at the end of stimulation and then rebounds to the normal course of the *c*-wave. That the factor causing this downward notch is removed by light adaptation can be seen by comparing *B* and *C*, obtained from the same "fast" eye (Fig. 2). Although both responses are very near the base line at the time of removal of the stimulating light, the response at *B*, with the eye light adapted, shows no tendency to form a downward notch. However, the *d*-wave, which is characteristic of the light-adapted eye, is present in this response.

Changes in the form and amplitude of the response during the course of progressive dark adaptation were studied by means of short test flashes given at various intervals after the end of a 20-minute period of light adaptation. Nine series were completed using 0.002-second flashes and seven using 0.01-second flashes, the results being essentially the same with test flashes of either length. A representative series is shown in Figure 3. Light adaptation was found to cause a marked decrease in the amplitudes of the *a*-, *b*-, and *c*-waves and in the total duration of the response. The *c*-wave is most drastically reduced, and its recovery with progressive dark adaptation is largely responsible for the development of the prolonged negative condition characteristic of the dark-adapted eye.

With progressive dark adaptation the *b*-wave increases in an orderly manner, and its change in amplitude with time has been plotted in Figures 4 and 5. In Figure 4 the height of the *b*-wave (as percentage of the control height with the eye completely dark adapted) is plotted against the time of dark adaptation for a series from one eye. This is a typical series, and the flattening of the curve at *X* is a constant characteristic. This plateau of the curve, however, is really due to the action of a component of the *a*-wave and will be discussed in that connection. Its position with respect to time varies somewhat from one individual to another, and consequently it is somewhat masked in the averaged results shown in Figure 5.

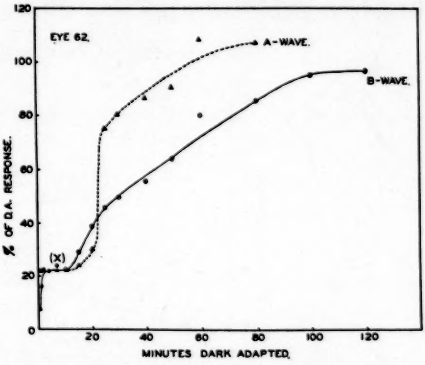


FIG. 4.—Amplitude of wave components (as percentage of the amplitude in the response of the completely dark-adapted eye) plotted against the length of time dark adapted.

From Figure 3 it will be seen that the *a*-wave remains relatively constant in amplitude during the early stages of progressive dark adaptation and then suddenly increases in amplitude, the increase taking place at about 20 minutes after the beginning of dark adaptation. A similar condition has been found in practically all of the series studied, although the increase may take place at any time between 15 and 45 minutes after the end of the period of light adaptation. This irregular behavior of the *a*-wave is due to the fact that this wave, as recorded from the dark-adapted eye, is

the resultant of the action of two components which become separated due to differences in their reaction to light adaptation. Their refusion accounts for the sudden increase in amplitude noted above. One of these components, which may be designated as the "migratory *a*-wave," is moved by light adaptation over onto the descending limb of the *b*-wave, while the remaining portion, the "stationary *a*-wave," is little affected either in amplitude or in position. Figure 6 shows the response to a series of 0.01-second flashes given at 0.75-second intervals. In this series it will be seen that the "migratory *a*-wave" is moved over onto the descending limb of the *b*-wave as the eye is progressively light adapted by the intermittent stimuli. A characteristic feature of the movement of this migratory component is the marked decrease in the amplitude of the *b*-wave when the "migratory *a*-wave" is superimposed on or near its peak, as is seen in the second response of this series.

The movement of this component may also be seen on the graph shown in Figure 4. Here the plateau in the curve and the irregularities shown between 2 and 10 minutes after the beginning of dark adaptation can be interpreted as due to the superposition of this notch on the peak of the *b*-wave. This represents the same type of reduction in amplitude as seen in Figure 6. As the "migratory *a*-wave" begins to move on down the ascending limb of the *b*-wave with progressive dark adaptation, the latter resumes its regular increase in amplitude. By comparing the curve for the amplitude of the *b*-wave with that for the *a*-wave (also Fig. 4) it will be seen that the amplitude of the latter is at first quite constant but then increases suddenly between 20 and 25 minutes after the beginning of dark adaptation or between 10 and 15 minutes after the migratory component has left the top of the *b*-wave. An examination of the records involved indicates that this interval represents the time occupied by the gradual movement of the "migratory *a*-wave" down the ascending limb of the *b*-wave. The sudden increase in amplitude of the *a*-wave is then the result of the fusion of its two components.

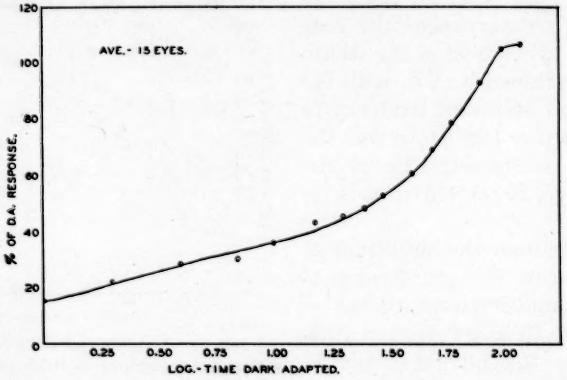
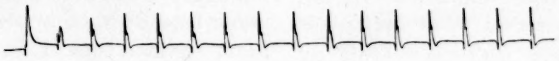


FIG. 5.—Amplitude of the *b*-wave (as percentage of the amplitude in the response of the completely dark-adapted eye) plotted against the logarithm of the length of time dark adapted. Averaged results from fifteen series.

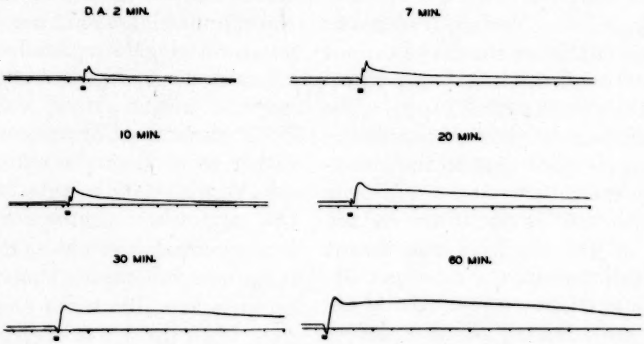
Records taken with a faster-moving paper (Fig. 7) show that the movement

of the "migratory *a*-wave" is due to two factors: a change in the latent period of the migratory-notch factor and a change in the slope of the ascending limb of the *b*-wave upon which the migratory notch is expressed. With progressive dark adaptation the latency of the notch decreases, varying in the series shown from



GALLERIA M. EYE 58. 0.01-SEC. FLASHES AT 0.75-SEC. INTERVALS.
TIME IN 0.2 SEC. TEMP.-25°C.

FIG. 6.—Responses of a "fast" eye to a series of 0.01-second exposures given at 0.75-second intervals



GALLERIA M., EYE 72, TEMP.-25°C., TIME IN 0.2 SEC., 0.04 SEC. EXP.

FIG. 7.—Responses to a series of 0.04-second exposures given at various intervals after the beginning of dark adaptation. Time of dark adaptation printed above each record.

52 sigma after 2 minutes of dark adaptation to 25 sigma after 30 minutes of dark adaptation. Accompanying this change, there is a decrease in the rate of rise of the *b*-wave which also tends to move the *a*-wave notch down its ascending limb.

DISCUSSION

The presence, in the response of the bee-moth eye, of all of the components (*a*-, *b*-, *c*-, and *d*-waves) considered as typical for the vertebrate electroretinogram distinguishes this response from that of any other arthropod so far studied. Accordingly, it is particularly suitable for a comparison with the vertebrate electroretinogram, and an attempt has been made to interpret the response of this invertebrate in terms of the three-component theory which Granit has proposed in connection with the vertebrate response. As mentioned above, all components of the electrical response of the bee-moth eye are opposite in sign to the analogous ones in the vertebrate electroretinogram. Brücke and Garten (1907) suggested that such a difference which exists between invertebrates and vertebrates is related to the inversion of the vertebrate retina.

With the exception of the relatively few cases of "fast" eyes, the *c*-wave of

the bee-moth response (eye dark adapted) is remarkably long, having a duration of several seconds for a very brief exposure, as, for example, in Figure 1, *A*. During continuous exposure to light for several seconds the *c*-wave is maintained near whatever peak it is capable of exhibiting in accordance with the degree of dark adaptation (Fig. 2, *E*). The existence of such long slow waves emphasizes the advisability of using a direct-coupled amplifier for the investigation of the responses of eyes of this type. Jahn and Crescitelli (1939) observed that the *c*-wave from the nocturnal Cecropia moth was of long duration.

It was found that certain features of the response of the bee-moth eye cannot be reconciled with the interesting three-component theory of Granit (1933). The nature and behavior of the *d*-wave is one of these points. According to the analysis made on the cat's eye by Granit, the *d*-wave at the "off" is due to the sudden extinction of the negative component (P III), which permits the more persisting positive component (P I) to be expressed as an increased positive deflection. In view of Granit's analysis, the following possibilities should be taken into account in attempting to explain the cause underlying the disappearance of the *d*-wave with dark adaptation.

There might be such a change in the time relationships of the antagonistic components as to balance out the effect of the extinction of the P III component. However, in this case there would be a prolonged interval between the end of stimulation and the beginning of the return of the response to the base line, unless the rate of extinction of all components were greatly increased. This latter is highly improbable, since dark adaptation is known to slow down all features of the response. An additional possibility is that there might be a decrease in the rate of extinction of the P III component so that its effect would be largely, but not necessarily completely, masked by the more rapid decline of the P I and P II components. Any residual effect of the action of the P III component would then appear as a delayed hump in the return of the response to the base line. Neither of these possibilities appears to apply to the disappearance of the *d*-wave, while the eye of *Galleria* is progressively dark adapted. As it disappears, there is no apparent shifting of the relationship of the *d*-wave to the "off" of the stimulating light, nor is there any notch or irregularity while the response subsides, such as might be attributed to a decrease in the rate of extinction of a P III component, if present. It appears rather to be a simple extinction of the *d*-wave with dark adaptation. However, this cannot be an extinction of the same component that produces the *a*-wave, as is the case in Granit's analysis, since the latter is actually at its greatest magnitude when the eye is dark adapted.

The mere presence of a *d*-wave in the response from the light-adapted eye of this moth is of interest, as the *d*-wave has previously been considered to be a distinct feature of the so-called "day" type of eye as exemplified by the grasshopper; and it was reported by Jahn and Crescitelli (1939) to be completely absent from the "night" type of eye as studied in the Cecropia moth. In their records the *d*-wave was absent even after long exposures to light.

Several lines of evidence indicate that the "migratory *a*-wave" is a distinct feature of the response of the bee-moth eye.

It can be followed to the point where it actually fuses with and enhances the "stationary *a*-wave," showing that it is a true positive component and not merely a lag between two phases of the rising negativity of the *b*-wave. Also, it causes a marked decrease in the amplitude of the *b*-wave when superimposed upon it (Fig. 6), indicating action positive in sign. From the results with short flashes it might be expected that the "migratory *a*-wave" is associated with the off-effect, but this is definitely not the case, since it is still present when series of 1.0-second flashes are employed. In such a series the "off" comes much later than the migratory notch. It is thus indicated that in the case of the compound eye of *Galleria* there are two different factors or components, each tending to produce a positive response to illumination and varying in their reaction to progressive light adaptation. However, Granit's theory provides for only a single negative component (polarity comparable to a positive component in the invertebrate response) and is thus incapable of accounting for the observations mentioned above.

Exactly what modifications in the theory are necessary to account for all of the features of the electrical response of the eye of *Galleria* has not been determined. A biological separation of the various components, of the general nature obtained by Granit (1933) using the cat's eye, will be necessary in order to obtain this information.

SUMMARY

1. The wave forms of the action potentials from the eye of the moth *G. mellonella* under various conditions of adaptation and employing different durations of stimulation by light have been described.

2. Light adaptation was found to reduce the amplitude of the *a*-, *b*-, and *c*-waves and also the duration of the total response.

3. The *a*-wave, as seen in the response of the dark-adapted eye, was found to be the resultant of the action of two components which may become separated from each other, owing to differences in their reaction to light adaptation. One component, the "migratory *a*-wave," is moved over onto the descending limb of the *b*-wave by light adaptation, while the remaining portion, the "stationary *a*-wave," is little affected by light adaptation.

4. Curves of the height of the *b*-wave for a constant stimulus plotted against time of dark adaptation and the logarithm of this time are presented to indicate the course of dark adaptation of this eye. Dark adaptation was usually found to be complete within 2 hours after 20 minutes of light adaptation, using an illumination of 290 foot-candles.

5. The *c*-wave, even for brief exposures, lasts for several seconds, its duration being much greater than that of any other component described.

6. The *d*-wave was found to be a characteristic feature of the response of the light-adapted eye and to disappear gradually with progressive dark adaptation. This disappearance is apparently a simple extinction of this phase of the response rather than a masking by another component.

7. The results of these experiments are discussed as indicating the inapplicability, without modification, of Granit's three-component theory to the behavior of the insect eye as exemplified by *G. mellonella*.

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NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

A SYMPOSIUM ON RESPIRATORY ENZYMES[1]

EDITED BY P. W. WILSON

Reviewed by C. G. KING

Nutrition Foundation, Inc., New York

IN THE sciences of biochemistry, physiology, and nutrition no area of research has been more fruitful in recent years than the study of respiratory enzymes. Fields of study that were apparently unrelated only a decade ago, except in the sense of dealing with living organisms, almost suddenly became "common ground." The isolation of vitamins by one group of investigators, the isolation of enzymes by a second group, and the study of oxidation-reduction reactions by a more or less independent third group, finally provided clear-cut patterns of reaction that the chemist could follow quantitatively in living cells. It is indeed doubtful whether any other decade has witnessed so many fundamental discoveries in relation to living cells.

The symposium at the University of Wisconsin, complementing the symposium on vitamins at the University of Chicago (published in a companion volume), provides an excellent summary of the present stage of progress in the study of respiratory enzymes. Since these catalysts control most of the reactions that constitute "intermediate metabolism," the book provides a valuable outline of the basic principles of physiology.

The papers are well organized with respect to coverage of major specific fields, and each chapter contains a critically selected bibliography. The authors have prepared excellent diagrams and sketches to correlate the respective reaction systems.

Informal photographs of many of the participants, taken at the time of the conference, add an element of human interest.

The level of writing is distinctly for the advanced student of biochemistry and physiology, though the better students in the related medical and biological sciences will be able to follow the articles with good understanding and interest. The quality of printing and editing is highly satisfactory.

Perhaps the value of the book and its timeliness can be indicated by the suggestion that university and college teachers in the biological sciences who are not familiar with its contents probably will be doing an injustice to their students.

[1] Madison, Wis.: University of Wisconsin Press, 1942. Pp. 281. $3.00.

THE BIOLOGICAL ACTION OF THE VITAMINS[1]
EDITED BY E. A. EVANS, JR.

Reviewed by C. G. KING
Nutrition Foundation, Inc., New York

EACH of the fourteen chapters in the book is based upon a symposium paper presented at the University of Chicago. The following titles and authors will indicate the scope of subject matter covered and indirectly will provide, for those who know the authors, assurance of the quality of the material presented: "The Biological Action of the Vitamins," by C. A. Elvehjem, Ph.D.; "Cocarboxylase," by S. Ochoa, Ph.D.; "Vitamin B_1: Clinical Aspects," by Norman Jolliffe, M.D.; "Riboflavin," by Paul Gyorgy, M.D.; "Human Riboflavin Deficiency (Ariboflavinosis)," by W. H. Sebrell, M.D.; "The Story of Pellagra and Its Treatment with Nicotinic Acid," by David T. Smith, M.D.; "Pyridoxine," by S. Lepkovsky, Ph.D.; "Pantothenic Acid and the Microbiological Approach to the Study of Vitamins," by Roger J. Williams, Ph.D., D.Sc.; "Pantothenic Acid in Human Nutrition," by Edgar S. Gordon, M.D.; "Biotin," by Vincent du Vigneaud, Ph.D.; "Choline," by Wendell H. Griffith, Ph.D.; "The Economy of Phosphorus in the Animal Organism," by Franklin C. McLean, Ph.D., M.D.; "Vitamin K," by D. W. MacCorquodale, Ph.D.; "Vitamin K: Clinical Aspects," by H. P. Smith, M.D., and E. D. Warner, M.D.

The papers have been prepared with evident care, both in their historical development of the respective subjects, including references, and in their consideration of reader-interest. The printing and editing show commendable workmanship.

Teachers, advanced students, physiologists, physicians, and others who attempt to keep abreast of research developments in biochemistry and nutrition will enjoy the book and will find it useful.

[1] Chicago: University of Chicago Press, 1942. Pp. 248+30 figs. $3.00.

BIOLOGICAL SYMPOSIA, VOLUME VIII: *LEVELS OF INTEGRATION IN BIOLOGICAL AND SOCIAL SYSTEMS*,[1] EDITED BY ROBERT REDFIELD

Reviewed by C. M. CHILD
Stanford University

THIS volume of the *Biological Symposia* consists of the following chapters: Introduction, Robert Redfield; "The Transition from the Unicellular to the Multicellular Individual," L. H. Hyman; "Intermediate Levels of Organismic Integration," J. W. Buchanan; "Higher Levels of Integration," R. W. Gerard; "Synergistic Aspects of Bacterial Populations," William Burrows; "The Transition from the Individual to the Social Level," H. S. Jennings; "Integration in Infra-social Insect Populations," Thomas Park; "Social Dominance and Subordination among Vertebrates," W. C. Allee; "Basic Comparisons of Human and Insect Societies," A. E. Emerson; "Societies of Monkeys

[1] Lancaster, Pa.: Jacques Cattell Press, 1942. Pp. v+240. $2.50.

and Apes," C. R. Carpenter; "The Societies of Primitive Man," A. L. Kroeber; "Modern Society," R. E. Park.

This symposium, held in connection with the celebration of the Fiftieth Anniversary of the University of Chicago, represents union of programs originally planned for separate symposia by biologists and the social science group. As is evident from the titles, the subjects considered range from the level of integration concerned in origin of multicellular from unicellular organisms to a discussion of modern society. Discussion in a single program of such widely different aspects of the problem of integration as is undertaken in this symposium is particularly interesting as an attempt to find, and actual discovery of, some common ground in different lines of research and thought. Authors differ in viewpoint and terminology and in their conclusions as to what is social and what is not. For example, Jennings apparently regards any influence of a living organism on another as social in character, while for Thomas Park the population pressures, some of which are such influences, are infrasocial in an insect population; and for Kroeber, Carpenter, and R. E. Park the term "social" undoubtedly has a different connotation. Redfield remarks that if we accept Jennings' definition of "social" the relation of predator and prey is social.

Buchanan holds that in the most highly integrated organisms all local independence is suppressed. This seems a rather extreme view. There appears to be a considerable degree of local functional independence of parts, even in the human body. Dominance in man is not a tyranny but approaches more nearly a sort of representative government based on data received from the various parts. Gerard also holds that increasing integration involves increasing subordination, both in the organism and in society; but R. E. Park maintains that freedom of the individual is greater in modern than in earlier society. Redfield suggests that government may extend its control over a greater number of units without necessarily exercising a greater degree of control, and that, so far as human society is concerned, different levels of integration may be involved with greater subordination at the ecological-economic-symbiotic level and greater freedom at the moral and religious level, as societal integration increases. But most of R. E. Park's illustrations of greater freedom in modern society have little relation to the moral and religious level. May it not be rather that the increasing subordination is concerned with the individual's position in the social system, and the greater freedom with his functional activities in that position. At the higher levels of physiological integration the organism shows something of this sort; the structure of the part is highly stable, but within the limits of that structure a considerable degree of independence of function appears.

Only a few of the many other points of interest in the contributions can be noted. In the Introduction Redfield discusses the program in general and order of presentation of papers, but the more important part of the Introduction consists of a survey of each paper with comments and suggestions on the points of greatest interest to him, on the differences in viewpoint of the authors, and on the implications of the symposium as a whole. Unfortunately, most of these must be left for the reader of the whole, and they are best read in connection with the reading of each paper.

Hyman, considering the various theories, concludes that origin of multicellu-

lar forms from flagellate protozoa is indicated by the evidence and regards transverse, or in the egg, equatorial cell division as an important factor in development of polarity and differentiation, though not present in *Volvox* and related forms. Polarity originates in effects of external differentials. It is perhaps pertinent to point out that transverse cell division is very generally characteristic of ciliates and that considerable development of polarity is found in many protozoa without relation to cell division.

Buchanan discusses development and nature of dominance in the individual, with experimental data on different degrees of integration, and notes that dominant regions are in general more active physiological systems, usually with higher oxidative metabolism, than other parts; but whether oxidative metabolism is the fundamental factor in dominance is not yet certain. Gerard, concerned with the further development of dominance and subordination, calls attention, first, to the evolutionary development of transmission of impulses as an integrating factor; second, in discussion of part-and-whole relations he gives us the concept "org," a unit system composed of lesser units with reciprocal relations between parts and whole; animorgs are living things. Is the concept "org" really useful as distinguished from "system"?

Burrows points out that, except for certain morphological aspects, "the individual bacterial cell has character only so far as it contributes to the properties exhibited by the population." There may be heterogeneity in the pure culture, and mixed cultures of different bacterial species may produce results of which none of the species is capable alone. Jennings regards allelocatalysis and aggregation in reaction to their own metabolic products as examples of integration without organization but finds in selective conjugation of different mating types of *Paramecium* a social organization of considerable complexity.

Thomas, Park centers attention on population size and finds that in consequence of the various "population pressures" an insect population becomes a complicated integrated system which has properties different from those of individuals, for example, birth rate and death rate. In comparing insect and human societies Emerson finds certain identities in the integrating factors. He notes that evolution of human society has been much more rapid than that of insects because repetition of social patterns occurs by means of the symbols which constitute social heredity and does not involve the germ plasm. Through social heredity we arrive at an ethical system as a social co-ordinating factor, and Emerson holds that scientific method is applicable to the study of this as of other social factors. Granting this, science does not stop when values are involved.

In the papers of Allee, Carpenter, Kroeber, and R. E. Park the integrating factors are psychological. In Allee's data on social order in birds, based on peck-right and peck-dominance and in mice on general aggressiveness, the level of integration is higher in birds, since it involves recognition of individuals. The experimental modifications of the order by hormone treatment and by conditioning by repeated victories or defeats are particularly interesting. High rank in the order has survival value, since it provides better conditions for reproduction. Carpenter discusses the organization of heterosexual social groups of various species of monkeys and apes with intra- and intergroup relations of dominance and subordination, usually with a dominant male at the head of the organiza-

tion and with position of individual and group maintained by competition. The group possesses definite territory and does not tolerate another group of the same species within it. Kroeber points out that among primitive peoples the structure of society is in general based primarily on relations of kinship, which are not recognized abstractly by animals, though kinship forms the basis of certain animal societies. Some of the modifications and extensions of this kinship factor are extremely complex elaborations—true luxury products. In the absence of any considerable political organization, cultural interests and energies apparently have been largely concerned with social structure based on real or artificial kinship. Some of our present-day fraternal organizations, clubs, and societies for this and that seem to the reviewer to be somewhat similar luxury products.

The different levels of integration—economic, political and moral—are, according to R. E. Park, only relatively independent, and it is probably their relations that make modern, or any other, society unique. Modern society differs from earlier in greater size, complexity, speed, mechanization, and freedom. If greater freedom is a feature of modern society, the National Socialist society of Germany is a regression, as often pointed out, and our own wartime society is becoming progressively less modern. Park holds that the fact that men still make war for a way of life suggests that societal integration is not declining or even completed. In this connection a comment of Redfield's is of interest:

> The common conscience and trust of scientists and scholars, of which this symposium is one small expression, is, in a wide sense, moral, and it is no small part of the way of life which many men will make war to preserve. The world of science and scholarship rests on qualities of common understanding that tempt one to look upon it as the beginning of an integration wider than has existed before.

A MANUAL OF EXPERIMENTAL EMBRYOLOGY[1]
BY VIKTOR HAMBURGER

Reviewed by VICTOR C. TWITTY
Stanford University

ALTHOUGH few subjects are more firmly established in the zoölogical curriculum than is descriptive embryology, the opportunity to illuminate this subject by instruction in the experimental analysis of development is seldom utilized. Even mere reference to well-established causal relationships is enlightening and stimulating to the student, but the impact and response are especially great when he can himself undertake experiments with living objects. Instruction in highly refined techniques need not be involved, nor contact with a wide variety of problems or organisms. Execution of a few selected experiments requiring only simple equipment will accomplish the principal objective, namely, to give the student a tangible appreciation of the dynamic aspects of development and the means by which they are studied.

A Manual of Experimental Embryology is well designed to facilitate and encourage such instruction. The experiments outlined have all been tested thoroughly

[1] Chicago: University of Chicago Press, 1942. Pp. 213. $2.50.

by the author himself in actual teaching practice and can be performed with facilities generally available in teaching laboratories or readily constructed by the student. Technical procedures are described in clear detail for each experiment separately, to permit flexibility in the laboratory schedule. The author also provides a classification of the experiments based on the degree of technical skill demanded of the student. The choice of living material—amphibians, chick, and flatworms—is in keeping with the wide procurability of these forms and their classical position as experimental objects. Altogether, the book will be found adaptable under a wide range of circumstances, including the degree of training and experience of instructor and student.

The *Manual* comprises five well-integrated sections: "Equipment and Instruments," "Experiments on Amphibian Embryos," "Experiments on the Chick Embryo," "Regeneration," and "The Gradient Theory." A total of sixty-five experiments is described, but in the Appendix the author proposes a selection from these designed to constitute a one-semester course.

The scope of the book is, however, not confined to a bare description of instruments and experimental procedures, as valuable as these alone will prove to be for both student and specialist. Although not designed as a text, it orients the student to each experiment by a brief introduction based on the pertinent literature and, through its well-chosen bibliographic references, enables the student to build further upon the lessons learned in the laboratory.

Special features which extend the usefulness of the *Manual* include: (1) well-executed drawings of the standard stage series of development in anurans and urodeles (copied from Shumway, Pollister and Moore, and Harrison); embryologists will be grateful to the author for making this material accessible under a single cover; (2) a chapter on the classification, life-history, and distribution of the principal species of Amphibia used for embryological experimentation, including instructions concerning their laboratory care, feeding, etc.; (3) an illustrated account of gastrulation in the urodele based on the work of Vogt and others by means of vital staining.

This latter feature, although it transgresses somewhat the formal scope of the *Manual*, is a particularly valuable addition. It provides, to my knowledge, the only suitable account available in English and may well be copied in texts of descriptive embryology in replacement of the traditional but outmoded version of early amphibian development.

The choice of experiments and animal objects, although arbitrary, is designed to provide a fairly broad view "in cross-section" of the classical procedures and results of experimental embryology. It does not follow that individual instructors choosing the *Manual* need observe in all cases precisely the same selection offered by the author. However, even those who because of their special interests or local circumstances may depart rather widely from the plan of the book will find it rich in suggestions and information valuable both to themselves and to their students.

The *Manual* contains several text drawings, mostly in illustration of technical procedures and instruments, a bibliography for each section, and an Index. It is published in attractive and durable form.